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No. 17.

ASCORBIC ACID.

PART 2: FACTORS DETERMINING STABILITY IN AQUEOUS SOLUTION.

By EINHART KAWERAU and W. R. FEARON.

[Read November 24, 1942. Published separately January 27, 1944.]

Although the conditions that bring about inactivation of Vitamin C have been widely recognised and investigated (Sherman, 1921; Clark, 1934; King, 1936, 1941), they have not hitherto been classified according to their relative importance. Ascorbic acid loses its antiscorbutic potency when the cyclic structure of the molecule is changed by the opening up of the lactone-ring. This does not occur readily in acid solutions, and the vitamin may be oxidised to the dehydro-form, and quantitatively recovered by the use of appropriate reducing agents. In alkaline solutions, however, the lactone-ring is spontaneously opened, with formation of diketogulonic acid, which subsequently breaks down to L-threonic and oxalic acids, from which the vitamin cannot be regenerated by simple reduction. Oxidation of the vitamin can occur, even in the absence of molecular oxygen, provided the solution is activated by: (1) ultra-violet radiation (Arcus and Zilva, 1940); (2) copper ions (Hess and Unger, 1921; Lindow *et al.*, 1929; Barron *et al.*, 1935, 1936; Spruyt and Vogelsang, 1938; McCarthy *et al.*, 1939; Tadokoro and Takasugi, 1939, 1939a; Mystkowski, 1942); (3) pyridine nucleotides (Tadokoro and Takasugi, 1939); and (4) enzymes of the "ascorbic oxidase" type (Szent Györgyi, 1928; Hopkins and Morgan, 1936; Stotz *et al.*, 1937; Srinivasan, 1937; Johnson and Zilva, 1937; Stone, 1937; Meiklejohn and Stewart, 1941).

Vitamin C Protectors.

From a survey of these observations, and taking into account the fact that both ascorbic acid and its primary oxidation product, dehydroascorbic acid, are of equal value in nutrition, a classification can be made of the methods available for protecting the vitamin from destruction following oxidation. Four classes of protectors are of special importance, namely: (1) substances capable of stabilising the vitamin by combining with it either in its reduced or oxidised form; (2) Reducing agents capable of maintaining the vitamin in the stable reduced form; (3) buffers that keep the solvent from becoming

alkaline; and (4) substances that act as anti-catalysts, either by depressing the ionisation of copper present in the solution, or by combining with "ascorbic oxidases."

1. *Vitamin Stabilisers.*—Although Scarborough and Stewart (1937) claim that ascorbic esters occur in urine, little is known definitely about such compounds (Fearon and Kawerau, 1943), apart from metallic derivatives such as those used in the original isolation of the vitamin (Szent Györgyi, 1928) but of no significance in the normal diet. Dehydroascorbic acid, however, has been shown to form compounds with glutathione (Bersin *et al.*, 1935), amino acids (Abderhalden, 1938), and thiols, in general (Drake *et al.*, 1942). Results for thiourea are given in Table 1, which shows the iodine titration values of fresh mixtures containing dehydroascorbic acid in buffered solution.

TABLE 1.—Stabilisation of Dehydroascorbic Acid by Thiourea.

Dehydroascorbic Acid: 50·0 mg. per 100 ml. In Borate Buffer, pH 6·5–7·0.
Thiourea: M/100. *Iodine*: N/100. *Temperature*: 15° C.

Dehydroascorbic Acid, in ml. A	Thiourea in ml. B	Iodine Value for A + B, in ml.	Difference of Iodine Value, in ml., due to A
0·00	1·00	0·95	——
1·00	1·00	0·70	0·25
0·00	2·00	1·68	——
2·00	2·00	1·26	0·42

NOTE: Dehydroascorbic Acid alone does not titrate with Iodine.

These data show that thiourea is capable of rapidly combining with and protecting dehydroascorbic acid in aqueous solution. Similar protection has already been found to be given by other thiol compounds (Drake, Smythe and King, 1942).

2. *Reducing Agents.*—At least three reagents are known to be capable of reducing dehydroascorbic acid, and keeping it reduced in ordinary aqueous solutions, namely: (1) hydrogen sulphide (Szent-Györgyi, 1928); (2) stannous chloride in absence of free oxygen (Meiklejohn and Stewart, 1941); and (3) glutathione in presence of tissue extracts (Mawson, 1935; Hopkins and Morgan, 1936; Crook, 1941). We have not been able to confirm the claim (Jorissen, 1941) that glucose in neutral solution reduces dehydroascorbic acid in absence of free oxygen. Other reducing substances capable of protecting ascorbic acid, such as uric acid and thiol compounds, appear to work in virtue of their anticatalytic rather than their reducing properties.

3. *Acid Buffers.*—Owing to the stability of ascorbic acid in solutions of low pH, inorganic acids have been widely used to extract the vitamin from its natural sources, but, as Mapson (1941) has shown, many samples of these acids, even after repeated purification, still contain sufficient copper to catalyse the extracted vitamin. Metaphosphoric acid, however, in high concentration completely depresses the ionisation of copper (Hinsberg, 1937), and for this reason is now generally employed as an extractant. Its instability is its first disadvantage, as on keeping it changes into the relatively ineffective orthophosphoric acid, a transformation that can be retarded for at least 15 days by keeping the solutions at 5° C. (Bessey, 1938). Another disadvantage comes from the fact that ferrous iron increases in reducing potential when in presence of metaphosphoric acid, and will bleach the indicator 2 : 6-dichlorophenolindophenol used in estimating ascorbic acid. This may be overcome by adding citric acid to the metaphosphoric acid, when extracting materials rich in iron (Lugg, 1942).

4. *Anti-catalysts.*—Reduced ascorbic acid is stable in tissue fluids and in natural and processed food materials containing appreciable quantities of copper. This stability is due to the presence of substances that form copper compounds of a low degree of ionisation. Such substances include: glutathione (Hopkins, 1929); thioglycollic acid—β-amino naphthalide (Berg, 1935); sodium diethyl-dithiocarbamate (Eden and Green, 1940); thiourea (Kohnschütter, 1903; McCarthy *et al.*, 1939); proteins and amino acids (Abderhalden and Schnitzler, 1927, 1927a; Borsook and Thimann, 1932); 8-hydroxyquinoline (Barron *et al.*, 1936; Sendroy and Miller, 1939); purines (Giri and Krishnamurthy, 1941); and halogen salts (Mapson, 1941).

Since copper is an invariable micro-constituent of all plant and animal tissues investigated, one or more natural protectors must be available in the organism to preserve the vitamin from catalytic destruction. If this be so, it should be possible to find a non-toxic substance with a high protective value, and capable of being applied both to the stabilisation of the vitamin within the organism and also in industrial preparations and food products. This was investigated by estimating the effect of various possible inhibitors on the catalytic oxidation of ascorbic acid in presence of known concentrations of copper. The first group of substances tested included thiourea and the chief non-colloidal solutes of normal blood plasma. The reagents employed were all of "analytically pure" quality, and the water was "glass-distilled." To suppress the error arising from varying contamination of the reagents by unavoidable traces of copper, and the necessity for frequent estimations, the amount of copper added in each experiment was at least a thousand times greater than that specified to be present in the regents. The reaction of the mixtures was kept at the specified levels, pH 6·4, and pH 6·8, by borate-phosphate buffers, checked from time to time. The ascorbic acid used was in crystalline form, as supplied by Messrs. Roche, and was estimated both by iodine and by iron titration (Fearon and Kawerau, 1943).

TABLE 2.—Protection of Ascorbic Acid from Oxidation in Presence of Copper.

Ascorbic Acid: 17·5 mg. per 100 ml. *Copper*: 2·5 mg. Cu^{++} per 100 ml. *Substances tested*: M/100. *Iron standard*: 1 ml. (= 0·1 mg. Fe^{+++}) is equivalent to 0·157 mg. ascorbic acid. *Temperature*: 15° C. *Reaction*: pH 6·4.

Substance tested.	Ascorbic Acid in mg. per 100 ml. At start	24 hours	48 hours	48 hours + Cu^{++}
Glass-distilled water ...	17·5	4·4	0·0	0·0
Urea	17·5	13·8	9·4	4·1
Uric acid	17·5	10·0	2·5	1·2
Glucose	17·5	9·7	4·1	3·1
Creatinine	17·5	15·1	3·1	3·1
Glutamic acid	17·5	10·0	4·1	0·9
Thiourea	17·5	17·5	17·3	17·3

The total volume of each mixture at the start of an experiment was 11 ml. At the end of 24 hours' incubation, each mixture was divided into two equal parts, to one of which distilled water containing the Cu^{++} was added, the other being diluted to the same extent with distilled water alone.

Control experiments showed that none of the substances tested had any reducing effect on the iron standard used in the titrations, both at the start and at the end of 48 hours' incubation.

A similar set of experiments was carried out in which the substances tested were present in concentrations of the same order as that found in normal blood plasma. In addition, the protective effects of hesperidin and of thiourea were examined.

TABLE 3.—Protection of Ascorbic Acid from Oxidation in Presence of Copper.

Ascorbic Acid: 35·0 mg. per 100 ml. *Copper*: 1·25 mg. Cu^{++} per 100 ml. *Temperature*: 15° C. *Reaction*: pH 6·8. *Total volume of mixture*: 20·0 ml.

Substance tested.	Concentration in mixture, %	Ascorbic Acid, in mg. per 100 ml. at start	after 24 hours	% recovery
Sodium chloride ...	0·5	35·0	0·0	0·0
Glucose	0·1	35·0	4·7	13·4
Urea	0·03	35·0	3·1	8·9
Glutamic acid ...	0·073	35·0	11·8	33·7
Uric acid	0·005	35·0	7·3	20·8
Creatinine	0·000,5	35·0	19·6	56·0
Distilled water ...		35·0	0·0	0·0
Hesperidin	0·007	35·0	0·0	0·0
Thiourea	0·006	35·0	35·0	100·0

Notes on the Protective Substances.

Sodium Chloride.—In concentrations of the same order as those of blood plasma, sodium chloride has no significant protective effect. To obtain this, it is necessary to have the solution more acid than pH 6 (Mapson, 1941; Mystkowski,

1942), while to attain 100 per cent. protection, sodium chloride concentrations of 12 per cent. are required (Mystkowski and Lasocka, 1939).

Glucose.—At the level of normal blood concentration, glucose is moderately effective, but the property is not a characteristic of the monosaccharide, and similar results have been reported for maltose and sucrose (Wokes *et al.*, 1942). A series of experiments carried out with glucose in presence of insulin, in which the hormone was in the normal blood concentration of 0·01 unit per 100 ml. (Gellhorn *et al.*, 1941), and also in concentration of 1 unit per 100 ml., gave no indication of any enhancement of the glucose protection effect.

Urea.—Protection can be demonstrated at concentrations similar to that in blood, but is too slight to be of obvious significance.

Creatinine.—Giri and Krishnamurthy (1941) report protection up to 100 per cent. in short-time experiments at 30° C. A similar degree of protection is provided by some purines, notably xanthine and uric acid. Creatine is ineffective. Our results are in conformity with these observations, and support the conclusion that creatinine and some of the purines, together with glutathione, are the chief non-colloidal protectors of ascorbic acid in animal tissues.

Hesperidin.—Some of the physiological effects of the as yet unidentified vitamin P may be explained in terms of ascorbic acid protection within the organism. Since hesperidin accompanies, or forms part of, the vitamin P complex, we investigated its protective action, but found it completely ineffective.

Glutamic Acid.—This amino acid was used in concentration equivalent to that of the total amino nitrogen of the blood plasma, and at this level afforded relatively high protection. Mystkowski and Lasocka (1939) report similar results for glycine, leucine, and aspartic acid. The property is probably possessed by all the amino acids and peptides, and is to be referred to their ability to form copper complexes of a low degree of ionisation. The nature of these complexes has been studied by Borsook and Thimann (1932), who find that in approximately neutral solutions, the copper is united to the oxygen of the carboxyl group by primary valencies, and to the nitrogen of the amino group by secondary valencies.

Proteins.—Although the ability of copper to catalyse the oxidation of ascorbic acid is lost when the metal is bound to amino acids or simple peptides, it is retained in some degree when the metal is combined with a protein. The combination usually is feeble, and the copper ions, Cu^{+} and Cu^{++}, can be removed by dialysis (Stotz *et al.*, 1937): conversely, proteins can immobilise the copper ions in a solution, and thus contribute to the protection of ascorbic acid to some extent.

Thiourea.—Of all the simple solutes examined, thiourea was outstanding in its protective power, and gave 100 per cent. in both sets of experiments, a result in confirmation of our earlier work on this compound. Thiourea has not been detected in the animal body. It appears to be of very low toxicity, and may find therapeutic application in circumstances where ascorbic acid stabilisation is required, such as during wound repair (Fearon, 1942).

STABILISATION OF ASCORBIC ACID IN INDUSTRIAL FOOD PREPARATIONS.

The chief food products rich in ascorbic acid are those made from citrus and other fruits, hence the problem of the stabilisation of the vitamin is of practical value in the domestic and industrial preparation of jam, marmalade, and similar preserves.

The possible significance of copper-contamination during processing has been recognised in an order by the British Food Ministry, according to which manufacturers of fruit concentrates containing vitamin C are required to satisfy the Ministry that their process-plants are constructed so that copper surfaces do not come in contact with the fruit juices (Charley, 1942).

The destructive effect of metallic copper and copper oxide on ascorbic acid is shown in the series of experiments recorded in Table 4. Five gram quantities of copper, in the form of bright turnings 2 mm. broad, copper oxide powder, or copper oxide sticks 5 mm. in diameter, were suspended in 100 ml. of a solution containing 46·0 mg. ascorbic acid, the concentration of which was estimated by titrations over a period of 300 minutes. The reaction of the mixture was pH 6·4, and the temperature was 14·0° C.

TABLE 4.

	Percentage Ascorbic Acid recovered.		
Time in minutes	Copper foil	Oxide sticks	Oxide powder
0	100	100	100
15	91·1	97·6	66·6
30	85·5	96·6	35·5
60	75·5	88·8	0·0
300	5·5	22·2	0·0

These results suggest that bright copper surfaces are relatively inert at low temperature, and that it may be sufficient for industrial purposes to ensure that the boiling pans are not lined by copper, the effect of copper parts of the "cold" sections of the plant being disregarded. Contamination by ionic copper is likely to be much more troublesome, since the metal is introduced in the water, and carried by the fruit, which is often copper-sprayed. Hence, the problem becomes one of finding an ascorbic acid preservative that will immobilise catalytic metals already present. McFarlane (1936) was the first to suggest that certain reagents used in the estimation of copper and iron might be employed commercially for the protection of vitamin C. He showed that the ascorbic acid content of orange juice suffered no loss by spontaneous oxidation if aa'-dipyridyl were added, to combine with the iron, and sodium diethyl dithiocarbamate, to combine with the copper. To be of commercial value, any such protector must conform to four requirements: (1) It must be non-toxic, and harmless to the consumer, over long periods, even in relatively high concentration; (2) it should be tasteless, odourless, and colourless, in the concentrations necessary; (3) complex-formation with the catalytic metals must

take place readily over a wide range of hydrogen ion concentration; (4) the complexes must be stable when exposed to the high temperatures and other conditions of the manufacturing processes.

These requirements greatly restrict the choice of protectors suitable for industrial use, and, in consequence, our attention was directed to only one of them, namely thiourea, as being likely to be of use. Unlike the copper complex formed with diethyl dithiocarbamate the thiourea-copper complex is colourless. In high concentrations, it separates out as a white, silky crystalline precipitate; in the low concentrations used in our experiments, no visible change is seen. For complete fixation of the copper, the thiourea must be present in excess; the ratio being 5 to 1 for copper sulphate (Rathke, 1884), and 3 to 1 for copper chloride (Kohnschütter, 1903). The results indicate that thiourea readily forms a copper complex in cold solutions, both at neutrality and in a high concentration of acid.

The value of thiourea as an anti-oxidant has long been known, and the reagent has been used to keep apples from turning brown on exposure to air (Denny, 1935, 1942). Thiourea is odourless, colourless, and without taste, when in dilute solution. Concentrated solutions have a bitter taste, but there is a considerable degree of difference in the susceptibility of different subjects to the taste of solutions of the same concentration. Feeding experiments carried out at the Boyce Thompson Institute and elsewhere have shown that thiourea is non-toxic to lower animals (Hanzlik and Irvine, 1921; Flinn and Geary, 1942). Medes (1937) reports that thiourea is excreted by the kidney in an unchanged form after administration to human subjects. Ingestion of 0·82 g., led to no detectable disturbance in metabolism, except for a slight rise in the urinary output of cystine. Hartzell (1940) reports that a single dose of 0·0168 g. resulted in no unpleasant symptoms. This evidence for the non-toxicity of thiourea has been confirmed by one of us (E.K.) for doses of one gram. Apparently, thiourea displays none of the pharmacological effects possessed by the substituted thioureas studied by Fastier and Smirk (1943). More work obviously is required on the effect of prolonged administration, and the possible formation of decomposition products during prolonged boiling with fruit extracts, before the general use of thiourea as a protector of vitamin C in large-scale industrial work can be advocated, but of all the compounds investigated it appears to be the most promising.

Natural Thermostable Protectors.—Since some vitamin C survives the various manufacturing processes employed in preserve-making, the presence of natural thermostable protectors is probable. Their existence was first demonstrated by Giri and Krishnamurthy (1940) in vegetable extracts from which ascorbic oxidase had been removed by acetone precipitation. We have observed that boiled vegetable extracts display a similar power of protection, which in some preparations is so complete as to suggest the presence of specific stabilisers, possibly of the thiol class.

Mercaptans have been detected in the distillate obtained by boiling cauliflower and red cabbage (Niemann, 1893), and, according to Rekowski (1893),

may arise by acid hydrolysis of more complex organic sulphur compounds. From the distillate yielded by boiled cabbage, Bach (1942) has obtained a sulphur-containing fraction soluble in light petroleum, and capable of acting as a vitamin C protector. Allyl isothiocyanate and sinigrin were found to act similarly in concentrations as low as $5{\cdot}7 \times 10^{-4}$ at pH 5·9. No attempt, however, was made to estimate or control the amount of copper present in the mixtures.

This work was extended by studying the relative protective effects of distillates from cabbage and potato, as shown in Table 5. Cabbage distillates contain a volatile thiol compound, the amounts of which were too small for exact identification. The compound can be characterised by the fact that it gives a transient violet colour on treatment with nitroprusside and ammonium hydroxide after saturating the solution with ammonium sulphate (Heffter, 1908), while the vapour from the solution at room temperature produces a brown stain on a filter paper moistened with lead acetate. The distillate bleaches standard iodine solutions, and at pH 2 titrates sharply with 2 : 6-dichlorophenol indophenol in a manner similar to ascorbic acid. This observation may explain the alleged four-fold increase in ascorbic acid content that Ahmad (1935) has claimed when cabbage has been boiled for 10 minutes.

Autolysis of cabbage extract at 37° C. for 18 hours does not set free volatile thiols, which is in support of Rekowski's contention that acid hydrolysis is required for their release. Potato juice retains some of its protective properties after boiling, and also yields an active distillate. The factor is present both in the surface layer and skin and also in the central part of the tuber. Unlike cabbage, the distillate gives no thiol reactions, although it slowly bleaches iodine. During the boiling of potatoes the protective factor readily passes into the water, whereas during the boiling of cabbage, it escapes with the steam.

A hundred grams of fresh cabbage leaves were boiled with 250 ml. of tap water. From the time of boiling, the distillate was collected over a period of 30 minutes. The yield, 70 ml., was then diluted to 200 ml., and tested for thiol compounds, as described. For the experiments on ascorbic acid protection, the stock was further diluted 1 : 2 with the buffer solution containing the amounts of ascorbic acid and copper, as shown in the Table.

TABLE 5.—Ascorbic Acid Protection by Vegetable Juices and Distillates.

Ascorbic Acid: 46 mg. per 100 ml. *Temperature*: 14° C. *Reaction*: pH 4·6. Results expressed as percentage of the original ascorbic acid recovered after 18 hours. Corrections have been made for the reducing value of the protective substance added.

Copper,	Cabbage Extract.		Potato Extract.	
mg./100 ml.	boiled juice	distillate	boiled juice	distillate
0·25	——	——	66·3	27·5
0·50	53·3	43·4	44·6	24·4
	64·2	95·2		
5·00	22·3	00·0		

SUMMARY.

1. A classification of the agents capable of protecting ascorbic acid has been made, and their relative efficacy is discussed.

2. The protective effect of the chief non-colloidal solutes of blood plasma has been investigated.

3. The protective effect of thiourea in dilute solution has been found to be greater over a wide range of conditions than any other agent examined. The use of thiourea in the stabilisation of vitamin C is advocated.

4. Problems connected with the industrial preparation of fruit and vegetable products rich in vitamin C are discussed.

5. Volatile thiol compounds have been detected in the distillates obtained when cabbage is boiled, and their value as protectors of ascorbic acid has been confirmed.

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REFERENCES.

(1) ABDERHALDEN, E. (1938).—Fermentforschung, **15**, 522.
(2) ABDERHALDEN, E., and SCHNITZLER, E. (1927).—Z. physiol. Chem., **163**, 94.
(3) ABDERHALDEN, E., and SCHNITZLER, E. (1927a).—Z. physiol. Chem., **164**, 37.
(4) AHMAD, B. (1935).—Biochem. J., **29**, 275.
(5) ARCUS, C. L., and ZILVA, S. S. (1940).—Biochem. J., **34**, 66.
(6) BACH, E. (1942).—Z. f. Vitaminforschung, **12**, 289.
(7) BARRON, E. S. G., et al. (1935).—J. Biol. Chem., **112**, 625.
(8) BARRON, E. S. G., et al. (1936).—J. Biol. Chem., **116**, 563.
(9) BERG, R. (1935).—Ber., **68**, 404.
(10) BERSIN, T., et al. (1935).—Z. physiol. Chem., **235**, 12.
(11) BESSEY, O. A. (1938).—J. Biol. Chem., **126**, 771.
(12) BORSOOK, H., and THIMANN, K. V. (1932).—J. Biol. Chem., 98, 671.
(13) CHARLEY, V. L. S. (1942).—Food Manufacture, **17**, 8.
(14) CLARK, W. M. (1934).—Medicine, **13**, 207.
(15) CROOK, E. M. (1941).—Biochem. J., **35**, 226.
(16) DENNY, F. E. (1935).—Contr. Boyce Thomp. Inst., **7**, 55.
(17) DENNY, F. E. (1942).—Contr. Boyce Thomp. Inst., **12**, 309.
(18) DRAKE, B. B., SMYTHE, C. V., and KING, C. G., (1942).—J. Biol. Chem., **143**, 89.
(19) EDEN, A., and GREEN, H. H. (1940).—Biochem. J., **34**, 1202.
(20) EISLER, B., ROSDAHL, K. G., and THEORELL, H. (1936).—Biochem. Z., **286**, 435.
(21) FASTIER, F. N., and SMIRK, F. H. (1943).—J. Physiol., **101**, 379.
(22) FEARON, W. R. (1942).—British Medical J., II, 95.
(23) FEARON, W. R., and KAWERAU, E. (1943).—Scientific Proc. Royal Dub. Soc., **23**, 103.

(24) Flinn, F. G., and Geary, J. M. (1940).—Contr. Boyce Thomp. Inst., **11**, 241.
(25) Fujita, A., and Iwatake, D. (1935).—Biochem. Z., **277**, 293.
(26) Gellhorn, E., Feldman, T., and Allen, A. (1941).—Am. J. Physiol., **133**, 193.
(27) Giri, K. V., and Krishnamurthy, P. V. (1940).—Nature, **146**, 99.
(28) Giri, K. V., and Krishnamurthy, P. V. (1941).—Nature, **147**, 59.
(29) Hanzlik, P. J., and Irvine, A. (1921).—J. Pharm. Exp. Th., **17**, 349.
(30) Hartzell, A. (1940).—Contr. Boyce Thomp. Inst., **11**, 249.
(30a) Heffter, A. (1908).—Maly's Jahresberichte.
(31) Hess, A. F., and Unger, L. J. (1921).—Proc. Soc. Exp. Biol., **19**, 119.
(32) Hinsberg, K. (1937).—Biochem. Z., **290**, 125.
(33) Hopkins, F. G. (1929).—J. Biol. Chem., **84**, 269.
(34) Hopkins, F. G., and Morgan, E. J. (1936).—Biochem. J., **30**, 1446.
(35) Johnson, S. W., and Zilva, S. S. (1937).—Biochem. J., **31**, 438.
(36) Jorissen (1941).—Chem. Weekblad, **38**, 646.
(37) King, C. G. (1936).—Physiol. Reviews, **16**, 238.
(38) King, C. G. (1941).—Ind. Eng. Chem., Anal. Ed., **13**, 225.
(39) Kohschütter, V. (1903).—Ber., **36**, 1151.
(40) Lindow, C. W., Elvehjem, C. H., and Peterson, W. H. (1929).—J. Biol. Chem., **82**, 465.
(41) Lugg, J. W. H. (1942).—Austr. J. Exp. Biol., **20**, 273.
(42) Mapson, L. W. (1941).—Biochem. J., **35**, 1332.
(43) Mawson, C. A. (1935).—Biochem. J., **29**, 569.
(44) McCarthy, J. F., Green, L. F., and King, C. G. (1939).—J. Biol. Chem., **128**, 455.
(45) McFarlane, W. D. (1936).—Biochem. J., **30**, 1472.
(46) Medes, G. (1937).—Biochem. J., **31**, 1330.
(47) Meiklejohn, G. T., and Stewart, C. P. (1941).—Biochem. J., **35**, 761.
(48) Mystkowski, E. M., and Lasocka, D. (1939).—Biochem. J., **33**, 1460.
(49) Mystkowski, E. M. (1942).—Biochem. J., **36**, 494.
(50) Niemann, F. (1893).—Arch. Hyg., **19**, 136.
(51) Rathke, B. (1884).—Ber., **17**, 297.
(52) Rekowski, L. (1893).—Arch. Sc. Biol., **2**, 205.
(53) Scarborough, H., and Stewart, C. P. (1937).—Biochem. J., **31**, 2232.
(54) Sendroy, J., and Miller, B. F. (1939).—J. Clin. Investig., **18**, 135.
(55) Sherman, H. C. (1921).—Physiol. Reviews, **1**, 598.
(56) Spruyt and Vogelsang (1938).—Arch. Neerl. Physiol., **23**, 423.
(57) Srinivasan, M. (1937).—Biochem. J., **31**, 1524.
(58) Stone, W. (1937).—Biochem. J., **31**, 508.
(59) Stotz, E., Harrer, C. J., and King, C. G. (1937).—J. Biol. Chem., **119**, 511.
(60) Szent-Györgyi, A. von (1928).—Biochem. J., **22**, 1387.
(61) Tadokoro and Takasugi (1939).—Japan. J. Biochem., **60**, 835.
(62) Tadokoro and Takasugi (1939a).—Japan. J. Biochem., **60**, 929.
(63) Wokes, F., Johnson, E. H., Duncan, J., Organ, J. G., and Jacoby, F. C. (1942).—Qu. J. Pharm., **15**, 314.

No. 18.

ASCORBIC ACID.

PART 3: THE ASCORBIC ACID CONTENT OF FRUITS AND VEGETABLES GROWN IN EIRE.

By EINHART KAWERAU.

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In April, 1940, Fixsen and Roscoe published a series of tables of the vitamin content of human and animal foods. The figures very largely represent values obtained by estimations carried out by the authors themselves, as well as a selection of results chosen from over two hundred references to the world literature. Figures from Ireland are not included in this work of reference, because no data have hitherto been available. The work here presented was designed to fill this gap, and at the same time it was intended to show from what part of the dietary the Irish population received its main supply of vitamin C, in order that during emergency conditions this main supply might be adequately protected.

In connection with the task of estimating ascorbic acid in foods, two complex problems commonly arise. One concerns the methods of detecting and estimating the vitamin; the other the stability of the vitamin in its natural and in artificial environment. Earlier communications to this Society deal with these two problems, respectively (Fearon and Kawerau, 1943, 1944).

It has been already stated that the purpose of this investigation is twofold, and the following will serve to illuminate the issue more clearly. Vitamin C is produced during the growth period of the plant, and one's enquiry may be directed to estimate correctly the number of factors that determine the optimum production of the vitamin in a particular plant. This research, if it covers not one but a number of species, will supply answers to the following questions:—(1) What plants in this country contain the vitamin in the highest concentration? (2) At what time of the year is the highest concentration reached? (3) Does this period coincide with the best time for harvesting a particular plant? (4) Do the customary methods of tilling and soil treatment in this country influence the production of the vitamin favourably or adversely? All these questions relate to one type of enquiry; a second quite distinct line of enquiry would comprise the following questions:—Vitamin C is taken at every meal. Taking all quantitative and qualitative considerations into view, which is the main dietary source of the vitamin of the nation? What is the degree of destruction of the vitamin during transport and distribution to the

town consumer? How is it affected by home and commercial food processing? And, finally, what is the cheapest dietary source of the daily adult human requirements of the vitamin?

The limited facilities at our disposal have made it impossible to answer all the questions just set out. Where personal observations are lacking, and where it is permissible, reference will be made to results obtained by other workers in the same field.

Method of Enquiry.

The principles of representative sampling, as stated by Harris and Olliver (1942), were strictly adhered to where information concerning optimum plant production of the vitamin was desired. These principles include:—(*a*) if different plants are investigated, they should be grown on the same piece of ground; (*b*) if fruit at different stages of ripeness is selected, it should be picked from the same plant, or better still, from the same branch; and (*c*) a minimum of time should elapse between picking the plant, and carrying out the estimation. None of these principles need be observed when the dietary source of the vitamin is the chief concern. Random samples were selected from a number of shops (in Dublin), and the results were pooled. Where different methods of processing of food are studied, it is important to do a sufficiently large number of estimations in order to obtain significant average results characteristic of the particular process adopted.

Method of Analysis.

The considerations given in our first report (Fearon and Kawerau, 1, 1943) and the desire to make the results comparable with those of most other workers led me to choose titration with 2 : 6-dichlorophenol-indophenol, as the method used in this particular work. The procedure is as follows:—One side of a balance is modified so as to hold in suspension a 2 oz. mortar. The mortar is filled with 20 ml. of strong (16 per cent.) metaphosphoric acid that has been kept on ice. The balance is brought to equilibrium, and from 5 to 20 g. of the substance to be analysed cut with a horn spatula directly into the acid (if cutting is required). The amount taken is weighed, the mortar is removed from the balance, and the substance is thoroughly ground with a small quantity of washed quartz sand. The fluid is filtered off, the residue is returned to the mortar, and a second extraction is made. This then is also filtered, and finally the combined filtrates are made up to 100 ml. A quantity of this is taken, 1 ml. or more, according to the expected vitamin concentration, and is titrated against the dye. In cases where the extracts are strongly coloured, the method of Lanke (1939) is adopted; an interpolation titration is done in which the dye is extracted with a mixture of xylene and amyl alcohol for the determination of the end point. All estimations were carried out at least in duplicate.

Reagents.—(1) Glass-distilled water, iron- and copper-free.

(2) Metaphosphoric Acid (B.D.H.), a 16 per cent. aqueous solution, kept on ice. If the biological material is very rich in iron, the metaphosphoric acid should be made up in the following buffer :—citric acid 26·0 g., metaphosphoric acid 40·0 g., water 250 ml., under cooling add NaOH 12·0 g. (Lugg, 1942).

(3) 2 : 6-dichlorophenol-indophenol (B.D.H., Roche, Ltd.). Dissolve according to requirement, one to three tablets in 100 ml. of a borate buffer of pH 7·4. The solution on ice will keep its titre for 10 to 14 days, provided care is taken not to contaminate it. Where possible, the solution should be checked against pure ascorbic acid. Other methods for checking the dye are given in a previous communication (Fearon and Kawerau, 1943, 1).

(4) HCl, 36 per cent. If the buffered metaphosphoric acid is used, most extracts of biological materials require the addition of some strong acid in order to bring the pH to 1·5. It has been found sufficient to add 1·5 ml. of 36 per cent. HCl to the final extract.

In accordance with the introduction, the results are grouped under two main headings:—(1) Analysis of plant material freshly collected; and (2) Analysis of plant material collected on the Dublin grocery market; the latter includes analyses of plant material before and after different methods of processing.

Analysis of Plant Material Freshly Collected.

It will be seen from a glance at Table 1 that no attempt is made to give an entirely complete survey. Cultivated fruits were only analysed if they are consumed to a large extent by the population in the form of jam, and thereby constitute an important source of the vitamin in the dietary. Wild fruits were only analysed from the point of view that other abundant natural sources of the vitamin may be found similar to that already discovered in the rose hip. Except for the potato freshly picked, cultivated vegetables were not analysed, as it was thought that market samples would be sufficiently fresh and would yield more characteristic results as far as the Irish dietary is concerned.

The results given in Table 1 are all in agreement with those published by Fixsen and Roscoe. There is a considerable variance of opinion, however, on the effect of ripening on the vitamin C concentration in the fruit. For tomatoes, Wokes and Organ (1942) state that the vitamin content does not increase on ripening, and Andross (1941) maintains the same for rose hips, when saying that the under-ripe fruit has the highest vitamin value. In all my analyses, the unripe fruit was picked from the same plant as the ripe fruit, and there seems to be fairly uniform evidence that ripening increases the vitamin content; but it also becomes evident that the term "ripeness" requires definition; the cases of the medlar and the walnut are clear evidence in this respect. Accepting every-day terms, the unripe green walnut shell has more

vitamin C than the ripe fruit that is ready to discard the seed. The concentration is at its height just before the formation of the hard shell (M. Pyke, R. Melville and H. Sarson, 1942). The medlar, too, is not declared ripe until the fruit is soft and ready to discard the seed, and at this point its vitamin

TABLE 1.

Date of Analyses.	*Cultivated Fruit.*	Remarks	Vitamin C mg/100 g. of Fresh Fruit— Ripe.	Unripe.
15/7/42	Strawberries	Outdoor	58·6	—
15/7/42	Raspberries	„	30·9	20·1
15/7/42	Loganberries	„	33·1	21·3
10/9/42	Tomatoes	„	15·6	9·2
21/9/42	Tomatoes	„	28·2	—
21/9/42	Tomatoes	„	35·6	—
21/9/42	Tomatoes	Greenhouse	31·7	—
21/9/42	Tomatoes	„	16·6	—
	Wild Fruit.			
8/9/42	Blackberries		15·1	14·6
8/9/42	Haws		5·0	4·2
8/9/42	Sloes		4·8	1·8
20/11/42	Medlars	Ripe—Soft Unripe—Hard	0·2	13·5
23/10/42	Walnut Green Shell		669·8	—
23/10/42	Walnut Green Shell		550·0	—
23/10/42	Walnut White Kernel	Same Fruit	15·3	—
23/10/42	Walnut Hard Brown Shell		0·0	—
28/9/42	Wild Rose Hip	Co. Wicklow	415·8	241·7 (8/9/42)
25/9/42	Wild Rose Hip	Co. Dublin	211·2	—
25/9/42	Sweet Briar Hip	Co. Dublin	371·0	190·1
25/9/42	Burnet Rose Hip	Co. Dublin	267·0	—
17/9/43	Wild Rose Hip	Co. Kerry	365·3	—

content is low. From a biochemical point of view, we must recognise that the changes that occur in the flesh of the fruit just prior to the shedding of the seed are degenerative ones. Disintegration of cellular structure rapidly leads to loss of ascorbic acid, hence the "ripe" medlar hardly contains any of the vitamin. Ripeness might thus be defined as the state of the fruit at which maximum development has occurred prior to any preparation for the discarding of the seed. Where maximum development is not accompanied by obvious colour changes, as in the medlar, this point may be difficult to determine; where the fruit is naturally soft, like the tomato, degenerative changes may be impossible to discern at their early onset. One is inclined to believe, then, that this point of definition has an important bearing on the presentation of results.

The cultivated fruit may be considered under two headings: luxury and uitility fruit. Except for loganberries, only the latter class of fruit has been analysed, especially since most of the luxury fruit such as cherries, plums, peaches, etc., contain little of the vitamin. Strawberries and raspberries, although a luxury in the early season, are as a main crop chiefly used by the jam manufacturers, and hence must be considered in this connection with the Irish dietary. A similar statement can be made for the tomato, which is becoming less and less of a luxury food. Table 1 demonstrates the fact that there is no difference between outdoor and greenhouse-grown tomatoes as far as their vitamin C content is concerned.

Not all types of wild fruits from the hills and plains of the 26 counties have been analysed. Samples were collected from time to time, representing the main crops. Rose hips and blackberries must be considered the nation's most important natural store of vitamin C. The results indicate that it would be worth while, in case of shortage of this vitamin for medical purposes, to prepare vitamin concentrates from these sources (as has already been done in some countries). The walnut, although possessing a high vitamin concentration even at the time the nut can be collected, is too scarce a plant to contribute to the Irish dietary. The results given for the rose hips are significantly lower than those found by British workers. Species identification would have been important in this instance since Pyke and Melville (1942) have shown that the range of vitamin concentration is closely related to the species of rose. Further examination showed that the earlier the species ripened the higher was the vitamin concentration (Harrison, Blackburn and Bolton, 1942). According to Dr. D. A. Webb, the commonest varieties in Ireland are *R. canina* and *R. arvensis*. *R. micrantha* occurs in counties Cork and Kerry, and the Burnet Rose (*R. spinosissima*) grows along the coastal mountains; more rarely one finds *R. stylosa* and *R. agrestis*. Of these varieties *R. canina* shows the highest range of vitamin concentration, up to 1·0 per cent. in the tables of Pyke and Melville (1942). These data support the claim made by the Russian workers, Iwanoff and Bukin (1937), that the same species of rose shows a greater vitamin concentration if grown in a more northerly climate than in a southerly climate. Rose hips from Scotland have shown values of over 2·0 per cent., and some Russian varieties have as much as 4·0 per cent. The relatively moderate Irish values might find their explanation in the soft climatic conditions preponderant here; a sufficiently large number of samples have not been analysed, however, to justify an opinion on this point.

Other wild fruit not included in the tables might be worth collecting, if abundant in any particular region. Andross (1941) gives values for elderberries 25–30 mg. per 100 g. fresh fruit; bilberries 8–9 mg. per 100 g. fresh fruit; and crab apple 10–12 mg. per 100 g. of fresh fruit. Jam can be made from the berries of the sea buckthorn (*Hippophae rhamnoides*), which may contain as much as 200 mg. ascorbic acid per 100 g. of fresh fruit (Griebel and Hess, 1940), and is a plant native to the northern sea coast not far from Dublin, and along the banks of the Shannon near Athlone (Dr. D. A. Webb).

Analysis of Plant Material collected from the Dublin Market.

Dietary lack of vitamin C is most likely to occur during the latter half of the winter. Random samples of the more common vegetables were taken from the Dublin market, and analysed. Of those poor in vitamin C, only two samples were taken, whilst of those rich in the vitamin, at least 5–10 samples were analysed.

TABLE 2.—Market Vegetables.

Date of Analysis	Vegetable	Vitamin C mg./100 g. fresh plant. Average	Vitamin C mg./100 g. plant. Range given by Fixsen and Roscoe, 1940
2/10/42 18/1/43 3/12/42	Cabbages, all samples, Duke of York Varieties: Savoy and	50·5	20·0–60·0
18/1/43 20/1/43	Brussels Sprouts	70·5	72·0–146·0
20/1/43	Cauliflower	54·3	19–0–101·0
21/1/43	Turnip (yellow, Swede)	26·0	17·0–43·0
22/1/43	Turnip (white)	23·5	
25/1/43	Carrot	10·2	1·0–31·0
25/1/43	Celery	4·8	1·0–5·7
25/1/43	Onions	10·0	2·0–15·0
27/1/42	Horse Radish	27·0	
18/10/42	Kohlrabi, leaves	112·8	
18/10/42	Kohlrabi, tuber	65·2	16·0–100·0

The results in Table 2 are encouraging in that they all tend to reach the upper limit of the range given by Fixsen and Roscoe. For winter vegetables collected from the market, this is a very satisfactory result. The only vegetable included in the table that was not bought on the market is the kohlrabi, a plant unfortunately not grown on the field scale in Ireland. Warne (1942) also reported high vitamin C values for kohlrabi grown in England, and advocated field scale cultivation. This palatable vegetable certainly should be fostered. One has little hope, however, of changing the dietary of the Irish rural population.

ANALYSIS OF IRISH POTATOES.

Scurvy was well known in Ireland after the potato famines of the last century. Even at the present day, the lack of adequate supplies of cabbage in some of the rural districts of Ireland is astounding. To insure healthy potato crops, and an adequate and cheap supply during the early spring, has always been recognised as a policy of vital national importance.

Vitamin C in the potato might have been first suspected by Szent-Györgyi (1925), who obtained a strongly reducing fraction from potato juice three years before he isolated his now famous "hexuronic acid" from the adrenal cortex. Pietsch (1927) did not suspect the antiscorbutic vitamin, either, when he found that potato juice reduces o-dinitrobenzene, a reaction that is now known to be given by vitamin C (Fearon and Kawerau, 1a, 1943). The work of Pelc and Podzimková (1933) is amongst the first for the correct assay of vitamin C in the potato. Many extensive surveys were to follow (Pfankuch, 1935; Pett, 1936; Scheunert *et al.*, 1937; Smith and Paterson, 1937; Steward and Preston, 1940; Smith and Gillies, 1940; Olliver, 1941). War conditions have aroused fresh interest in the potato, as a nearly complete food (Salaman, 1940; Chick, 1940; Lampitt and Goldenberg, 1940).

Two major difficulties attend the assay of vitamin C in the potato by the dye reduction method. The first is described in the work of Smith and Paterson (1937), who have shown, contrary to the earlier work of Pfankuch (1935), that virus-diseased potatoes may contain up to over 50 per cent. more of the vitamin than healthy specimens of the same variety; they go even as far as to rely on vitamin assay for the diagnosis of virus infection. All potatoes analysed by me, except the random market samples, were obtained with the aid of the Department of Agriculture as Government certified virus-free. The second difficulty also arises from diversity of opinion. Pfankuch (1935) and Guthrie (1937) mention a glutathione concentration in the potato of under 10 mg. per 100 g., whereas Pett (1936) reports values as high as 80 mg. per 100 g., which would be 5–8 times as much as the usual average concentration of ascorbic acid, and thus would lead to erroneous values in the dye titration unless most stringent conditions are observed. I have tested a number of samples for glutathione by the procedure recommended by Lugg (1942), and have come to the conclusion that in the final dilutions of potato extract employed the glutathione constitutes a negligible fraction as far as the reduction of the dye is concerned.

Results for Raw Potatoes:—Table 3 shows a typical series of values obtained for the various parts of the potato plant (variety, Kerr's Pink) immediately on harvesting. The highest concentration of ascorbic acid occurs in the leaf of the plant, a fact also recorded by Smith and Gillies (1940); but unfortunately potato leaves are unsuited for human food. As far as the tubers are concerned, the highest concentration for this variety coincides with the tubers of the greatest fresh weight. The exhaustive research of Smith and Paterson (1937) into the vitamin C distribution of the potato gives consideration to the fresh weight of the tuber, but any corresponding relationship to the vitamin concentration is considered by them to be an inconstant and unimportant feature.

A survey including all potato varieties that are grown in Ireland would have been too extensive a work to be included in this survey; all varieties obtainable on the Dublin market were, however, analysed in December, 1942.

TABLE 3.—Freshly Harvested Potato (Kerr's Pink).

Date of Analysis	Part analysed	Total fresh weight. g.	Vitamin C mg./100 g.	Vitamin C mg/100 g. Smith and Gillies, 31/8/1940
11/9/42	Leaves	—	89·5	51·0
„	Stem	—	0·5	15/6/40 10·0–20·0
„	Tuber	85·0	34·0	31/8/40 31·0
„	Tuber	30·0	27·4	
„	Tuber	11·0	23·8	
„	Roots	—	1·0	

After visiting more than 20 different sources of supply, only four different varieties could be obtained. From these, the largest tubers were selected, each weighing over 130 g., and the results are given in Table 4.

TABLE 4.—Market Potatoes, December, 1942.

Variety	Vitamin C mg/100 g. Average of 5–10 Tubers	Vitamin C mg/100 g. Smith and Gillies, 1940
British Queen	17·32	
Kerr's Pink	21·30	12·90
Arran Pilot	14·1	
Arran Chief	13·5	5·70

The ascorbic acid content in potatoes at least 4 months old appears to be very good when compared with the figures of Smith and Gillies (1940). The higher results may be due entirely to the difference of size. Scheunert *et al.* (1936, 1937) in their potato analysis took into consideration the fresh weight of the tuber; unfortunately, however, they only classified them into "large" and "small" ones. For new potatoes, they found in every case a greater concentration of the vitamin in the larger potatoes; in old potatoes, they found this relationship to hold good only for some varieties. Table 5 shows the results of the analysis of a stone of *Kerr's Pink* after four months' storage,

TABLE 5.—Relation of Size to Vitamin Content.

Variety.	Weight in g.	Vitamin C mg./100 g.	Variety.	Weight in g.	Vitamin C mg./100 g.
British Queen	50–100	18·16	*Kerr's Pink*	50–100	13·1
	100–130	18·00		100–130	17·4
		—		130–150	21·3

and a stone of *British Queen* freshly harvested, the potatoes being assorted according to their weight.

The results are average values for an analysis of at least 5 tubers; in some weight groups, ten tubers were analysed. *Kerr's Pink* appears to show significantly greater values for heavy tubers, whether they are freshly harvested (Table 3) or whether they have been stored for four months; the same observation could not be made on *British Queen.* Drew and Deasy (1941), in their analysis of Irish potatoes for their starch content, showed that, speaking for the same variety of potato, a reduction of size resulted in an increased percentage of starch; it is not likely, therefore, that the increased vitamin concentration is correlated with the starch concentration.

A number of investigators (Smith and Paterson, 1937; Ott, 1937; Scheunert *et al.* 1937, 1940) found that climatic conditions and different methods of manuring had very little influence on the ascorbic acid concentration in the potato tuber. Root vegetables differ in this respect from fruit. Charley's observations (1942) on blackcurrants grown in controlled plots over a number of years indicate that climatic conditions may have a profound influence on the ascorbic acid content of the fruit; a similar statement has been recorded for rose hips.

Results for Cooked Potatoes.—The survival of vitamin C after varying methods of cooking has been a matter of extensive investigation. The position may be summarized as follows:—Ascorbic acid is fairly well protected in its natural surrounding, especially if the cellular contents include glutathione (Crook and Hopkins, 1938), as obtains in the potato. The vitamin survives freezing (Scheunert and Reschke, 1940), heating to 100° C. or over, as naturally occurs in frying (Lyons and Fellers, 1939), and even drying in air vats (Javillier, 1939; Somogyi, 1943). The amount of sodium bicarbonate commonly added to boiled vegetables does not appear to harm the vitamin (Olliver, 1940). A complete review of the various cooking methods, as given by Miss Olliver (1941), demonstrates as a general fact for all vegetables that the loss of ascorbic acid is one due to extraction by the cooking water, and not so much to actual chemical change. This significant statement is corroborated by the results obtained in my experiments on cooked potatoes. Potato tubers (British Queen), harvested in August, 1943, were sorted according to their fresh weight, and used for the following cooking experiments within the week of lifting. The method most commonly employed in the rural districts of Ireland for cooking potatoes was adhered to. The potatoes were put into cold water to which salt had been added, and were boiled until they became soft on being pricked by a fork, which usually occurred within 20–30 minutes. Peeling of potatoes was performed with a stainless-steel slotted-blade knife. At least four independent analyses in duplicate were made for each sample.

The principles that can be deduced from the results of Table 6 are generally applicable to most vegetables, except for one important difference: all green leafy vegetables contain an enzyme, ascorbic acid oxidase, which is capable of destroying the vitamin rapidly, so rapidly indeed, that, for example, in

the tomato 92 per cent. of the vitamin may be lost in 7 minutes from the time of slicing (Wokes and Organ, 1942). For this reason, green vegetables should be put directly into boiling water or live steam. The potato alone may be put on in cold water, since it does not contain the enzyme (Johnson and Zilva, 1937; Mommaerts, 1943). The more finely a vegetable is cut, the more of the vitamin is lost by enzyme action and by subsequent extraction into the cooking

TABLE 6 (*a*).—Cooked Potatoes.

Unpeeled and uncut before treatment.	Vitamin C mg./100 g.	Peeled and cut into quarters before treatment.	Vitamin C. mg./100 g.
Before Cooking (entire tuber)	17·78	Before Cooking (entire tuber)	18·55
Immediately after Cooking 20–30 minutes†	13·00	Immediately after Cooking 20–30 minutes†	8·90
After 4 Hours keeping in Hot-box†	10·30	Mashed and kept 4 Hours in Hot-Box†	3·30
24 Hours after Cooking, Air-cooled†	10·00	Mashed, kept 24 Hours, Air-cooled†	0·85
24 Hours after Cooking, re-heated to 50° C. for 15 min.	5·20	—	—
Original Cooking Water. (10 Tubers) pH 5·0–6·0	1·50–2·10	Original Cooking Water. (3 Tubers)	1·35–2·88

† Edible portion only.

TABLE 6 (*b*).—Cooked Potatoes. Balance Sheet.

Unpeeled and Uncut Potatoes.			Potatoes Peeled and Cut into Quarters.		
	Vitamin C			Vitamin C	
% Retained.	% Extracted.	% Destroyed.	% Retained.	% Extracted.	% Destroyed.
95·6	1·8	2·6	53·4	10·6	36·0

water (Wellington and Tressler, 1938). Heating beyond the minimum cooking time, keeping hot, and re-heating are practices to be avoided if waste of the vitamin must be prevented (Scheunert and Reschke, 1938; Olliver, 1940).

Occasionally the question is raised as to "bound ascorbic acid" in the potato. Considering the new chemical reaction for dehydroascorbic acid described by Fearon and Kawerau (1, 1a, 1943), the possibility of a bound ascorbic acid that has even at present failed to be identified, must be borne in mind. We are in agreement with Harris and Olliver (1942) and Rolf (1941) in saying that by the ordinary methods of boiling and weak acid hydrolysis, no "bound ascorbic acid" can be detected. Dehydroascorbic acid has been

found in the potato up to a concentration of 5·0 mg. per 100 g. of healthy tuber, and this value does not increase during storage at which time the reduced ascorbic acid decreases up to 50·0 per cent. of its original value (Smith and Gillies, 1940).

At times the question is raised as to how much of the vitamin is discarded with the potato peels. In Table 5 I reported on the vitamin content of Kerr's Pink potatoes, the amount of vitamin for the three weight groups ranged between 13·1 and 21·3 mg. per 100 g. Raw potatoes belonging to the identical weight groups were peeled as described, and the vitamin content of the very thin skins was estimated. For all three weight groups the vitamin content of the skins was practically identical, the range being: 8·0–9·0 mg. per 100 g.

ANALYSIS OF PRESERVED FOODS.

The next most important items as far as the vitamin C content of the Irish dietary is concerned, are jams. At some times of the year, particularly during the emergency, jam is more plentiful than butter, and must be considered an important supplement to the vitamin C intake, especially in young children. Methods of preserving food differ, and no matter how extensive a survey is made, the loss of the vitamin by destruction can never be accurately predicted unless every step is taken to secure optimum conditions for preserving the vitamin. The whole problem of protection of ascorbic acid has been dealt with in a previous paper, and for the present I wish only to emphasise one of the main items, namely, the significance of copper utensils used in preserving fruit. The organic matter of the fruit offers fair protection to the vitamin if traces of copper are involved, but the amount of copper removed by the fruit acid from a copper preserving pan is sufficient to destroy more than 50 per cent. of the ascorbic acid during the short period of heating. The results given in Table 7 make this point obvious; in both experiments the same crop of rose hips was used.

TABLE 7.—Vitamin Destruction by Copper. Wild Rose Hips.

Lot 1 in Copper Pan. *Method.*	Lot 2 in Enamelled Pan. *Method.*
Rose Hips 2 lb.; Sugar 1 lb. 2 oz. Hips washed and put into Copper Pan, covered with water and brought to boil and simmered until tender, about 20 min. Pulp was then pressed and residue again covered with water and brought to boil momentarily in Copper Pan. Pulp again pressed and juice returned to Copper Pan, when sugar was added and syrup boiled for 5 minutes.	Rose Hips 2 lb.; Sugar 1 lb. 2 oz. Hips washed and crushed and put into Enamelled Pan and covered with one pint of water. T = 70° C. Stood for 12 hours. A further pint of boiling water added and again stood for 12 hours. The mash was then pressed and the juice returned to Enamelled Pan and the sugar was added. The syrup was now boiled for 5 minutes. The final volume was the same as in Lot 1.
Ascorbic Acid: 35·2 mg/100 g.	Ascorbic Acid: 98·0 mg./100 g.

It has generally been claimed that where ideal conditions prevail, commercial methods of fruit preserving give better vitamin returns than home methods (Olliver, 1940; Lueck and Pilcher, 1941), especially where canning is resorted to. The final vitamin concentration must depend on the initial vitamin concentration; so if home preserving starts at the same level as commercial preserving, working with the same precautions it can achieve a higher final vitamin concentration, because during home preserving economic considerations need not restrict the final fruit concentration of the jam to approximately 60 per cent. The high ascorbic acid values given in Table 8 as

TABLE 8.—Home-made Fruit and Vegetable Extracts.

Date	Material analysed	Vitamin C mg/100 g.	Method of Preparation.
21/7/42	Syrup of Blackcurrants	49·3	10 oz. Water + 5 oz. Fresh Fruit. The Fruit is put straight into the quick boiling water; boiled 5 min., strained, no sugar.
21/7/42	Syrup of Blackcurrants	40·5	10 oz. Water + 5 oz. Fresh Fruit. Fruit crushed and left standing for 15 min., then treated as before.
17/7/42	Syrup of Blackcurrants	143·0	10 oz. Water + 56 oz. Market Fruit. Crushed and hot water added. Sterilized at 160° F. for 15–20 min.
17/7/42	Syrup of Blackcurrants	100·1	10 oz. Water + 56 oz. Market Fruit (different lot from previous sample). Crushed after heating at 160° F. for 15–20 min.
Oct. 1942	Syrup of Elderberries	13·2	1·0 oz. of Water to 3·0 oz. of Fruit. Boiled 3 min. Fruit crushed while coming to the boil. No sugar. Analysis 5 days after prep.
Oct. 1942	Juice of Cabbage	20·0	Boiled to softness with ½ quantity by weight of water. Analysis 3 days after prep.
Oct. 1942	Juice of Swede Turnip	0·4	Boiled to softness with ½ quantity by weight of water. Analysis 3 days after prep.

* Except for the vitamin analyses, these cooking experiments were carried out by Dr. Ella Webb.

compared with the low values given in Table 9 for the vitamin concentration in blackcurrant jam, must be interpreted on these lines.

TABLE 9.—Commercial Fruit and Vegetable Extracts.

Date	Material analysed	Vitamin C mg/100g.	Manufacturers	No. of Jars analysed
Oct. 1941	Orange Marmalade	9·3	A	6
Oct. 1941	Juice of Canned Whole Oranges	49·0	E	4
Oct. 1941	Juice of Canned Whole Carrots	3·7	E	2
Nov. 1941	Rose Hip Syrup	57·0	B	4
Nov. 1941	Rose Hip Syrup	47·2	C	2
Nov. 1941	Rose Hip Syrup	68·0	D	1
Nov. 1941	Rose Hip Syrup	67·3	A	3
Oct. 1942	Rose Hip Syrup	113·0	A	4
Oct. 1941	Blackcurrant Jam	25·2	A	4
Sept. 1942	Blackcurrant Jam	35·1	A	3
Sept. 1942	12 months old Canned Blackcurrant Purée	34·8	(English)	1

It is interesting to note in Table 8 that crushing of the fruit before heating results in loss of the vitamin, and how, by increasing the fruit concentration, values for ascorbic acid can be obtained that are far higher than those reached in commercial production.

Commercial rose hip concentrates in 1941 were very poor in their vitamin content when compared with the values, 100·0–200·0 mg. per 100 g., given in the English literature (Andross, 1941). The local manufacturers allowed me to investigate their process to determine at which stage the loss of vitamin occurred. Over 50 samples were analysed, and in accordance with the findings the process was modified, giving the satisfactory result in 1942 of an at least 50 per cent. increase in the vitamin content of Irish manufactured rose hip syrups.

The suggestion has been made that it might be necessary to dispense rose hip syrup through the hospital dispensaries, in case importation of the synthetic vitamin should fail altogether. The question would then arise as to how fast the vitamin deteriorated in an opened jar of rose hip syrup. It will seen from Table 10 that the loss after the first month is not more than 10 per cent.; practically 50 per cent. is lost after 4 months, and a slight amount of fermentation has set in after this length of open storage, a thing not likely to occur in busy dispensaries.

Unfortunately no data are available for the rate of deterioration of vitamin C in unopened jars of rose hip syrup. Wokes *et al.* (1942) compute the loss of ascorbic acid from rose hips as 1 per cent. per week at 5·0° C., with a coefficient of 2·0 for each 10·0° C. rise. At storage temperature of 15·0° C., the average, the loss therefore is only slightly greater than that in the fresh fruit.

TABLE 10.—Deterioration of Vitamin C on Keeping.

Description	Remark	Vitamin C Content in mg./100 g. Fresh	After One Month	After 2 M.	After 4 M.	After 12 M.	After 18 M.
Rose Hip Syrup (Firm A, 1941) 3 Jars analysed	Clear Glass Jars stored in cupboard (left open)	53·5	48·0	43·0	26·5	—	—
(% of Vitamin deteriorated)		—	(10·2%)	(19·5%)	(49·9%)	—	—
Rose Hip Jelly Firm A, 1942)	Jars as above (Only unopened Jars analysed)	61·1	—	—	—	12·0	—
(% of Vitamin deteriorated)		—	—	—	—	(80·3%)	—
Orange Marmalade (Firm A, 1941)	Jars as above (Only unopened Jars analysed)	9·3	—	—	8·5	—	5·0
(% of Vitamin deteriorated)		—	—	—	(8·6%)	—	46·2%

Analysis of the Cost of Vitamin C.

The vitamin C requirement of the healthy adult human being has been assessed by independent workers (Review: Brit. Med. J., 1942, p. 227), and is accepted at the level of 50 mg. per day. Based on this fact, a table was compiled by Miss Olliver in 1940 showing the cost of 50 mg. of vitamin C, as derived from various sources of cooked and preserved foods. A table of this nature, of course, is only of topical interest, since the cost of the different foods varies enormously with the seasons and with the general economic conditions of the country, especially during a state of emergency. Table 11 is similar to that of Miss Olliver's; where applicable her prices relating to the English market of the autumn of 1940 are included in the table.

TABLE 11.—Comparative Cost of Ascorbic Acid in Eire.

Material	Cost of 1 lb.	Vitamin C mg./100 gm.	Amount required to give 50 mg.	Vitamin C Cost of 50 mg.
Potatoes Boiled unskinned	1½d.	13·0	13 1/3 oz.	1d.
Potatoes Boiled skinned	1½d.	8·9	1 lb. 3½ oz.	1½d.
M. Olliver (1940) Potatoes Home cooked	1½d.	8·0	1 lb. 6 oz.	1½d.
Cabbage Juice	2d.	20·0	8¾ oz.	1d.
M. Olliver (1940) Cabbage Home cooked	2d.	21·0	8½ oz.	1d.
Elderberry Syrup	Sugar 5½d.	13·2	13 oz.	4¼d.
Home made Blackcurrant Jam	Fruit 1s. Sugar 5½d.	143·0	1 oz.	1d.
Commercial Blackcurrant Jam	11½d.	37·2	4½ oz.	2¾d.

TABLE 11—*continued.*

Material	Cost of 1 lb.	Vitamin C mg./100 gm.	Amount required to give 50 mg.	Vitamin C Cost of 50 mg.
Home Made Rose Hip Syrup	Sugar 5½d.	98·0	1¾ oz.	¼d.
Commercial Rose Hip Syrup	2s.	113·0	1½ oz.	2¼d.
Commercial Marmalade	1s. 7d.	9·3	1 lb. 2¾ oz.	1s. 9½d.
Tomatoes	2s.	28·2	6½ oz.	9¾d.
M. Olliver (1940) Tomatoes	10d.	24·0	7¼ oz.	4½d.
Synthetic Vitamin C	£27	One Tablet 50 mg.	One Tablet	¾d.

The calculations for the main food items, cabbage and potatoes, agree well with those of Miss Olliver. Clearly one does not often eat the amounts necessary of these foods, to obtain all the daily requirement, but in the rural districts where potatoes are always cooked in their skins and eaten in great quantities, no fear need be entertained as to inadequate supplies of the vitamin. The only source of the vitamin that equals the synthetic product in cheapness is home-made rose hip syrup. The notion that one can obtain all the vitamin necessary by taking a little marmalade daily (a popular idea) is a costly delusion, as the table well illustrates.

SUMMARY.

A limited survey of the ascorbic acid content of Irish grown Fruits (wild and cultivated) and Vegetables is presented.

Special attention has been paid to potatoes, rose hips, and blackcurrants.

The effect of cooking on the vitamin was studied by preparing potatoes in a variety of ways. For the better preservation of ascorbic acid, it was found that potatoes should be steamed or boiled in their skins; that most of the vitamin was lost if the potatoes were skinned previous to boiling, if they were re-heated or kept in a hot-box.

The effect of home and commercial preserving methods on the vitamin have been studied. The apparent advantage of home preserving is that a higher fruit concentration can be obtained. Wild fruits can be collected free, with the result that the jam, and with it the vitamin, costs little. The advantage of commercial preparations is founded in the better keeping properties of the material.

In conclusion I wish to express my gratitude to Professor W. R. Fearon for much helpful criticism and advice, and Dr. D. A. Webb and Dr. Ella Webb for their kind collaboration; also to Mr. T. D. McKeever, B.A., and Mr. J. Harwood of this department, for assisting with some of the estimations.

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BIBLIOGRAPHY.

(1) Andross, M. (1941).—Analyst, **66**, 358.
(2) Charley, V. L. S. (1942).—Food Manufacture, **17**, 8.
(3) Chick, H. (1940).—Chem. and Ind., **59**, 737.
(4) Crook, E. M., and Hopkins, F. G. (1938).—Biochem. J., **32**, 1356.
(5) Drew, J. P., and Deasy, D. (1941).—J. Dpt. Agr. (Eire), **38**, 220.
(6) Fearon, W. R., and Kawerau, E. (1943) (1).—Sc. Proc. Roy. Dublin Soc., **23**, 103.
(7) Fearon, W. R., and Kawerau, E. (1943) (1a).—Biochem. J., **37**, 326.
(8) Fearon, W. R., and Kawerau, E. (1944).—Sc. Proc. Roy. Dublin Soc., **23**, 171.
(9) Fixsen, M. A. B., and Roscoe, M. H. (1940).—Nutr. Abstracts and Reviews, **9**, 795.
(10) Griebel, C., and Hess, G. (1940).—Z. Unters. Lebensm. **79**, 469.
(11) Guthrie, J. D. (1937).—Contr. Boyce Thompson Inst., **9**, 17.
(12) Harris, L. J., and Olliver, M. (1942).—Biochem. J., **36**, 155.
(13) Harrison, J. W. H., Blackburn, K. B., and Bolton, E. (1942).—Nature, **150**, 574.
(14) Iwanoff, N. N., and Bukin, V. N. (1937).—Quoted by Pyke and Melville. (Cf. 29.)
(15) Javillier (1939).—C. R. Acad. Agric. France (per Nutr. Abstr., **10**, 336).
(16) Johnson, S. W., and Zilva, S. S. (1937).—Biochem. J., **31**, 438.
(17) Lanke, L. S. (1939).—Skand. Arch. Physiol., **81**, 300.
(18) Lampitt, L. H., and Goldenberg, N. (1940).—Chem. and Ind., **59**, 748.
(19) Lueck, R. H., and Pilcher, R. W. (1941).—Ind. Eng. Chem., **33**, 292.
(20) Lugg, J. W. H. (1942).—Austr. J. Exp. Biol., **20**, 273.
(21) Lyons, M. E., and Fellers, C. R. (1939).—Amer. Potato J. (per Nutr. Abstr., **10**, 100).
(21a) Mommaerts, W. F. H. M. (1943).—Z. Vitaminforschung, **13**, 250.
(22) Olliver, M. (1940).—Lancet, II, 190.
(23) Olliver, M. (1941).—Chem. and Ind., **60**, 586.
(24) Ott, M. (1937).—Angew. Chem., **50**, 75.
(25) Pelc, H., and Podzimková, M. (1933).—Nutr. Abstracts, **3**, 1003.
(26) Pett, L. B. (1936).—Biochem. J., **30**, 1228.
(27) Pfankuch, E. (1935).—Biochem. Z., **279**, 115.
(28) Pietsch, A. (1927).—Biochem. Z., **181**, 183.
(29) Pyke, M., and Melville, R. (1942).—Biochem. J., **36**, 336.
(30) Pyke, M., Melville, R., and Sarson, H. (1942).—Nature, **150**, 267.
(31) Rolf, L. A. (1941).—British Chem. and Physiol. Abstr., III, 181.
(32) Salaman, R. N. (1940).—Chem. and Ind., **59**, 735.
(33) Scheunert, A., Reschke, J., and Kohlemann, E. (1936).—Biochem. Z., **288**, 261.
(34) Scheunert, A., Reschke, J., and Kohlemann, E. (1937).—Biochem. Z., **290**, 313.
(35) Scheunert, A., and Reschke, J. (1938).—Nutr. Abstracts, **8**, 399.
(36) Scheunert, A., Reschke, J., and Kohlemann, E. (1940).—Biochem. Z., **305**, 1.
(37) Smith, A. M., and Paterson, W. Y. (1937).—Biochem. J., **31**, 1992.
(38) Smith, A. M., and Gillies, J. (1940).—Biochem. J., **34**, 1312.
(39) Somogyi, J. C. (1943).—Helv. Physiol. Acta, **1**, C, 50.
(40) Steward, F. C., and Preston, G. (1940).—Plant Physiol., **15**, 23.
(41) Szent-Györgyi, A. v. (1925).—Biochem. Z., **162**, 399.
(42) Warne, L. G. G. (1942).—British Med. J., I, 387.
(43) Wellington, M., and Tressler, D. K. (1938).—Nutr. Abstracts, **8**, 399.
(44) Wokes, F., and Organ, J. G. (1942).—Nature, **150**, 523.
(45) Wokes, F., Johnson, E. H., Duncan, J., Organ, J. G., and Jacoby, F. C (1942).—Qu. J. Pharm., **15**, 314.

No. 19.

THE CHEMICAL CONSTITUENTS OF LICHENS FOUND IN IRELAND.

CLADONIA SYLVATICA (L.) HARM. EMEND. SANDST.

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THE lichen *Cladonia sylvatica* (L.) Harm. emend. Sandst. was found growing abundantly on peaty soil on the summit of Howth Hill, Co. Dublin, and was identified for us by Mr. Mackenzie-Lamb, of the Natural History Section of the British Museum, to whom we wish to express our thanks. It bears a very strong resemblance to the lichen *Cladonia impexa* Harm., from which it is distinguished by the morphological feature of the tips being bent in one direction. While Asahina records (Acta Phytochimica, 1934, **8**, 52) that *Cladonia impexa* shows a negative reaction to benzidine and p-phenylene diamine, *Cladonia sylvatica* gives a red reaction with an alcoholic solution of p-phenylene diamine, indicating the probable presence of a depsidone of the salazic acid type.

The constituents of the lichen *Cladonia impexa* Harm. have already been described by us (Sci. Proc. R.D.S., 1942, **23**, 6). The predominant constituent is l-usnic acid; the lichen also contains the depside perlatolic acid and the sugar alcohol d-arabitol. There is also present a small amount of a sterol-like substance to which we attributed the alternative formulae $C_{32}H_{50}O_4$ or $C_{32}H_{52}O_4$. In view of the results now recorded in the case of the variety of *Cladonia sylvatica* we reinvestigated this sterol-like substance, and found it to consist principally of the triterpene acid, *ursolic acid*, $C_{30}H_{48}O_3$.

We find that in *Cladonia sylvatica* (L.) Harm. the principal constituents are usnic acid and ursolic acid; the latter was identified by comparison of it and its derivatives with ursolic acid and its derivatives, prepared from the leaves of the Bearberry plant (*Arctostaphylos uva ursi*) which grows extensively on the coast of Clare in the neighbourhood of Ballyvaughan. In contrast to the usnic acid from *Cladonia impexa*, which is pure l-usnic acid, the usnic acid in *Cladonia sylvatica* is only weakly lævo-rotatory, and consists principally of the inactive compound. Ursolic acid, which is a very minor constituent of *Cladonia impexa*, is present in *Cladonia sylvatica* in considerable amounts. On the other hand, the amount of perlatolic acid present in *Cladonia sylvatica* is very much less than in the case of *Cladonia impexa*. We did not succeed in isolating identifiable amounts of sugar alcohol from *Cladonia sylvatica*, but obtained in the acetone extract a small amount of what appeared to be fumaro-protocetraric acid.

EXPERIMENTAL.

Ether extract of lichen.

700 g. of air-dried lichen were rubbed through a $\frac{3}{8}''$ sieve and extracted twice with $2\frac{1}{2}$ litres of ether. The extract yielded 10 g. of a green gummy

mass, which was treated with 80 c.c. ether, giving an ether solution A_1 and a residue A_2.

The solution A_1 was shaken with 30 c.c. of 5 per cent. sodium bicarbonate solution; the alkaline solution was acidified, extracted with ether, and the product recovered from the ether crystallised several times from 60/80 petrol, when a very small amount of cream-coloured rosettes were obtained—m.p. 102° C. They gave a reddish-brown colour with ferric chloride, and a negative reaction with bleaching powder. A mixed melt with perlatolic acid showed no depression. The amount obtained was only a small fraction of that obtained from an equal weight of *Cladonia impexa.*

Analysis: 4·280 mg. gave 10·510 mg. CO_2; 2·700 mg. H_2O.
5·20 mg. gave 2·72 mg. AgI.
Found C = 67·0; H = 7·2; OCH_3 = 7·15.
$C_{24}H_{29}O_6 . OCH_3$ requires C = 67·5; H = 7·2; OCH_3 = 6·98.

The residue in the ether solution A_1 insoluble in sodium bicarbonate was an intractable gum which was not further investigated.

The residue A_2 was treated with 100 c.c. chloroform, giving a greenish yellow solution A_3 and a grey residue A_4. The solution was concentrated to 25 c.c. and 50 c.c. alcohol added. On standing yellow needles were deposited, which on recrystallisation from a mixture of choloroform and alcohol melted at 193° C. This product had a specific rotation in chloroform solution of −20·8, and was inactive usnic acid with a small admixture of the lævo variety.

Analysis: 3·973 mg. gave 9·08 mg. CO_2 ; 1·7 mg. H_2O.
Found C = 62·4; H = 4·75.
$C_{18}H_{16}O_7$ requires C = 62·8; H = 4·6.

The chloroform-insoluble fraction A_4 was washed with a small amount of chloroform to remove traces of usnic acid; it was then taken up in ether, and extracted with 5 per cent. sodium bicarbonate solution, which removed a small amount of material which, on crystallisation from 80 per cent. acetone, gave brown plates m.p. 220° C. with gas evolution. A similar product was found in *Cladonia impexa*; the amount was insufficient for further investigation. The ether solution of the partially purified A_4 gave a grey powder m.p. 240–250° C. which showed no reactions with bleaching powder or ferric chloride. It gave a positive Liebermann test, passing rapidly from purple to blue and finally green. It was dissolved in the minimum quantity of boiling alcohol, and on standing formed a gel which slowly transformed into white lustrous crystals, which were afterwards identified as ursolic acid. On shaking an ethereal solution with 10 per cent. caustic soda a white layer of sodium ursolate formed at the interface.

In order to obtain adequate amounts of ursolic acid 6¼ lbs. of air-dried lichen were extracted with ether; 60 g. of a yellowish green material were thus obtained. This was treated in lots of 25 g. as follows:—25 g. were warmed with 50 c.c. alcohol with vigorous stirring, and allowed to stand overnight. The whole was then filtered, washed with 50 c.c. alcohol, and finally

with 10 c.c. alcohol. The alcohol mother liquors contain most of the gum and perlatolic acid, while the alcohol-insoluble residue consists principally of usnic and ursolic acids. The dried residue (18 g.) was treated with successive quantities of 40 and 25 c.c. hot ligroin to remove some further gummy material and some usnic acid; the remainder of the usnic acid was then removed by boiling with 75 and 50 c.c. chloroform. In this way from the total 60 g. of original product, 36·5 g. of a grey powder were obtained. This was crystallised successively from alcohol and from benzene, when a product m.p. 272° C. was obtained, the mother liquors containing material melting below 250° C. The product was then further purified by sublimation in a high vacuum at 275° C.; the ursolic acid sublimed as a snow-white mass of crystals, a small amount of gummy material forming a more volatile fraction. The crystalline material was further purified by crystallisation from alcohol, and then melted at 280–282° C. This material was used for analysis and in the preparation of derivatives.

Analysis: (1) Air-dried material—
3·772 mg. gave 10·47 mg. CO_2; 3·49 mg. H_2O.
0·662 mg. – 5·846 mg. camphor; 8·8° △.
Found C = 75·9; H = 10·28; M.W. = 488.
$C_{30}H_{48}O_3 . H_2O$ requires C = 75·9; H = 10·55; M.W. = 474.

(2) Dried in vacuum at 110° C.—
3·671 mg. gave 10·62 mg. CO_2; 3·48 mg. H_2O.
Found C = 78·89; H = 10·53.
$C_{30}H_{48}O_3$ requires C = 78·94; H = 10·52.

Ursolic acid phenyl urethane.

0·3 g. ursolic acid was dissolved in 60 c.c. toluene, and to the solution 0·04 c.c. phenyl isocyanate in 2·5 c.c. toluene added; the whole was heated for 10 minutes at 100° C., and then concentrated to 10 c.c.; 25 c.c. warm ligroin were then added, and the white precipitate formed crystallised by dissolving in a small volume of chloroform and adding 6 volumes of ligroin. Large transparent plates of the urethane were obtained, m.p. 235° C. Before analysis the material was dried in vacuum at 110° C.

Analysis: 3·863 mg. gave 10·96 mg. CO_2; 3·26 mg. H_2O.
3·379 mg. gave 0·09 c.c. N at 753 mm. and 24° C.
Found C = 77·3; H = 9·3; N = 3·03.
$C_{37}H_{53}O_4N$ requires C = 77·24; H = 9·2; N = 2·43.

Methyl ursolate.

Methyl ursolate was prepared by interaction of sodium ursolate and methyl iodide according to the method of van der Haar (Rec. Trav. Pays Bas, 1924, **43**, 375, 4). It was also prepared from ursolic acid using diazomethane, silver oxide and methyl iodide, or sodium methoxide and methyl iodide. In all cases a gummy material was obtained, which on crystallisation from 60/80 petrol melted finally at 166° C. and showed no depression on mixing with methyl

ursolate prepared from ursolic acid isolated from the leaves of the Bearberry according to the method of Bilham, Kon, and Ross (J.C.S., 1942, **41**).

Analysis: 3·968 mg. gave 11·44 mg. CO_2; 3·88 mg. H_2O.
4·595 mg. gave 2·89 mg. AgI.
Found C = 78·6; H = 10·7; OCH_3 = 6·76.
$C_{30}H_{47}O_2 . OCH_3$ requires C = 79·15; H = 10·6; OCH_3 = 6·6.

Methyl acetyl ursolate.

0·2 g. of the methyl ester was heated for 1 hour with 0·2 g. sodium acetate and 3 c.c. acetic anhydride. On cooling, the mixture was treated with water, and the acetate recrystallised from alcohol. It melted at 235° C., and showed no depression on admixture with genuine methyl acetyl ursolate.

Diacetyl ursolic acid.

0·3 g. ursolic acid was heated for 1 hour with 3 c.c. acetic anhydride. On standing, a crystalline precipitate was formed, which was collected and dried for three days in vacuum over potash. The material melted at 198° C. and then resolidified, and did not melt again until 285° C. It gives a positive Liebermann reaction.

Analysis: 3·88 mg. gave 10·67 mg. CO_2; 3·29 mg. H_2O.
6·856 mg. required 2·3 c.c. N/100 NaOH.
Found C = 75·1; H = 9·4; Acetyl = 14·4.
$C_{30}H_{46}O_3 . (CH_3CO)_2$ requires C = 75·5; H = 9·6; Acetyl = 15·9.

Monoacetyl ursolic acid.

0·2 g. of the diacetyl compound was heated for 1 hour with 5 c.c. of 70 per cent. alcohol. On filtering and evaporating the alcohol to half bulk white crystals were obtained, which on crystallising from alcohol had m.p. 281° C. not depressed on admixture with monacetyl ursolic acid.

Analysis: 4·081 mg. gave 11·49 mg. CO_2; 3·67 mg. H_2O.
Found C = 77·18; H = 9·9.
$C_{30}H_{47}O_3 . (CH_3CO)$ requires C = 77·1; H = 10·0.

Acetone extract of lichen.

700 g. of lichen, after extraction with ether, were extracted with 2½ litres acetone, and gave 4·7 g. of a green paste; this was warmed with water and filtered. On treating the aqueous extract in the same way as in the case of our investigation of *Cladonia impexa* (*loc. cit.*) there was only a small residue, from which it was not possible to isolate any sugar alcohol. The water-insoluble fraction was warmed with 80 per cent. acetone, and filtered. The solution on standing deposited a small amount of crystals, which were soluble in sodium bicarbonate, gave a purple colour with ferric chloride, and a negative reaction with bleaching powder. They turned brown at 220/230° C., and charred at 260° C. and gave a sublimate of fumaric acid. They were probably fumaro-protocetraric acid.

No. 20.

SEA TROUT OF THE WATERVILLE (CURRANE) RIVER.

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Previous work on the sea trout of the Waterville River (Went and Barker, 1943) had raised two interesting problems connected with these fish. As explained by the authors the samples of sea trout examined were representative only of the spring sea trout runs, the close season for the capture of salmon and trout by means of nets and weirs in that river commencing on 16th July, before the smaller sea trout begin to run in force. The two problems arose from the fact that the calculated smolt length of these spring-running sea trout (mean length 10·4 inches) was exceedingly high, being some 3 or 4 inches longer than smolts from other Irish rivers investigated to date (Nall, 1929, 1930, and 1931). Had the smolts of the later-running sea trout similar high mean lengths was the first question to be solved, and, if so, were sea trout fry or smolts being taken by anglers before their initial descent to the sea in the belief that they were small adult sea trout?

In order to solve this problem arrangements were made for the collection of sets of scales and data from the rod catches of sea trout during the 1942 season.[1] Mr. P. J. Hamilton, Clerk and Inspector to the Waterville Board of Fishery Conservators, kindly undertook to carry out the work of collecting the necessary material. Most of the new material, comprising 845 sets of scales and data, was obtained from the rod catches when they were being displayed at the end of each day in the various hotels in Waterville, Co. Kerry. In this way about one-sixth of the total number of fish caught in 1942 by visiting anglers to Waterville was sampled. As far as possible the various sizes were selected for sampling roughly in the proportion in which they occurred in the catches. The majority of the fish captured by anglers in 1942 were taken from Lough Currane, the other lakes in the Waterville system only being fished to a limited extent owing to difficulties in transport. In Table 1 the distribution of the samples, both in time and locality, has been indicated.

[1] The investigation described by Went and Barker, 1943, was completed towards the end of 1941, but circumstances prevented the completion of the typescript and figures until late in 1942. Arrangements had been made, however, for the collection of the new material before the paper reached its final form.

Length of the smolts.

In order to simplify this report it is proposed to discuss matters somewhat out of their usual order, and to deal firstly with the calculated smolt lengths. As will be explained more fully in a later section the length at the end of every year of life of each maiden or unspawned fish with suitable scales was calculated together with the length of the smolt (see page 207). The mean length of all smolts was 9·85 inches, which is somewhat less than that calculated previously (10·4 inches) for the spring sea trout runs (Went and Barker, 1943, page 95). Nevertheless the order of the mean length of the smolts is the same as that obtained previously. Whilst it is true that the small sea trout (fish in their first post-migration summer) have a lower average smolt length than the earlier-running sea trout, the differences are relatively small. In the previous investigation, as in Nall's (1931), the scales of previously spawned fish were included for measurements, which in itself leads to somewhat inaccurate results. Naturally, when dealing with scales taken from rod-caught fish one is unable to tell the date of entry into a river of the individual fish in question. In order to ascertain whether there was one or more distinct groups or populations (the term "race" is hardly correct in this case) the frequency distribution curve of the smolt lengths for all maiden fish examined, including the small number investigated previously, was plotted (see Fig. 1). It is obvious from this curve that there was no clear differentiation, based on the length of the smolt, into two or more groups, in the sea trout of this river. This being so, the material from the spring-running sea trout described previously and the present material have been pooled, and the following portion of this paper relates to such pooled material.

In addition to the distribution curve for all smolts the individual curves for the length of smolts of fish captured in their first post-migration summer and fish captured at a later period of life (mainly those in their second post-migration summer) were added to Fig. 1. It will be seen that the maiden fish in their second post-migration summer have a somewhat greater mean smolt length than those in their first post-migration summer, the mean lengths being 10·2 inches and 9·7 inches, respectively. At this stage, however, it is advisable to mention that even the grouping of material does not give a true picture of the proportion of each class of fish, because of the variation in the percentage of fish sampled. For example, the catch of sea trout in the nets and weirs seldom reaches 1,000 in any one year, and the number of fish sampled was 433, say very approximately 50 per cent. of the total yearly catch. On the other hand one-sixth or less of the rod catch was sampled. By suitable arithmetical manipulation, however, the data available can be used to obtain a fairly accurate idea of the stocks of sea trout in this river, and in the majority of tables the weighted results have been given. (This has been accomplished by considering that the rod-caught fish were three times more numerous than they actually were in the samples.)

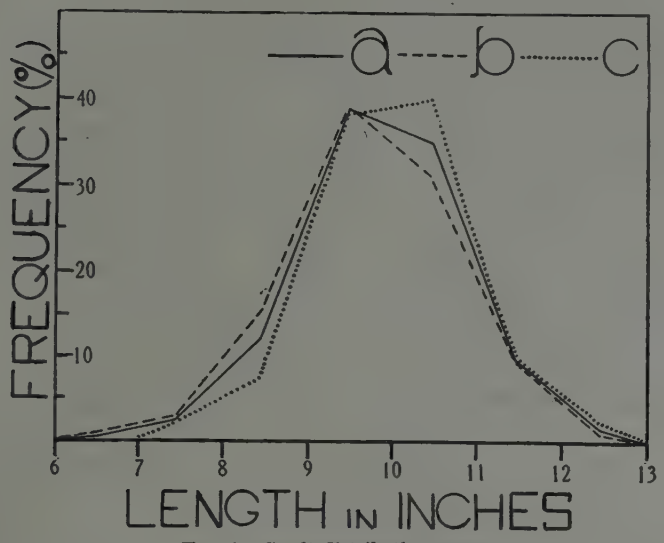


FIG. 1.—Smolt distribution curve.

(*a*) All smolts.
(*b*) Smolts of whitling (maiden fish in their first post-migration summer).
(*c*) Smolts of maiden fish in their second and third post-migration summers.

Are sea trout fry[2] or smolts being taken by anglers?

The question as to whether sea trout fry or smolts are being taken unwittingly by anglers in the belief that they are small adult sea trout is the second problem to be solved. Out of 845 sets of scales and data collected in 1942 there were, in addition to a few sets of scales unsuitable for microscopical examination, 39 sets taken from fish which showed on their scales no signs of sea feeding. A number of these fish were obviously brown trout, but there were other sets of scales which may have belonged to either the fry of sea trout or of brown trout. It is naturally impossible to tell from the scales to which category these fish belong, but in 25 cases there was the distinct possibility that the scales had been taken from sea trout fry.

The killing of these fry is, of course, contrary to the provisions of the fishery code, the capture of the fry or smolts of salmon and trout being prohibited by Section 73 of the Fisheries Act of 1842 (5 and 6 Vict. Cap. 106). It is difficult or impossible for an angler to ascertain whether the fish is actually a sea trout smolt without removing the scales. With the possibility of a smolt reaching a length of about 13 inches (the greatest calculated length in these investigations being 12·9 inches) it is only to be expected that the offence of taking sea trout fry might be occasionally committed. This difficulty is minimised to some extent by the enforcement of a by-law on the Waterville River, its

[2] The term "fry" is used here in the legal sense.

lakes and tributaries, prohibiting the killing of any sea trout or "pink" (the local name of sea trout fry) of a size less than nine inches from the tip of the snout to the fork of the tail (By-Law No. 276 dated 16th September, 1909. See Abstract of Fishery By-Laws, 1937). Apart from the fact that a technical offence is being committed by the taking of these large sea trout fry little real harm is being done to the fisheries when one considers the weight of fish involved. If one assumes that the smolts taken average 10 inches in length, and that on their first return to the river from the sea the same fish measure 15 inches, the relative weight of the adult fish to that of the smolt would be very roughly $1 \cdot 5^3$ to 1 or 3·4 to 1. This, however, ignores the possible mortality between the smolt and adult stage, so that taking into consideration the fact that relatively small numbers of these fish are probably taken each year little damage to the fisheries is likely to result therefrom. One cannot, however, apply this argument to the taking of sea trout fry in other rivers, since the average length of the sea trout smolts is usually 3 or 4 inches lower than that of the sea trout from the Waterville system.

Smolt ages.

The distribution of the various smolt classes has been given in Table 2. Four smolt ages, namely, two, three, four, and five-years were observed, the five-year smolts being only of slight importance, forming as they do just over one per cent. of the whole in the maiden fish. Two-year smolts comprised more than half of the total maiden fish examined, and the next most important group, the three-year smolts, were only two-thirds as numerous, forming 37·0 per cent. of the total. The smaller sea trout (i.e. fish in their first post-migration summer) had a greater proportion of old smolts than the larger fish. The two-year smolt class, the youngest group of smolts existing in this river, accounted for 73·3 per cent. of the fish of the second post-migration summer and older age groups, whereas in the first post-migration summer fish the percentage of two-year smolts was only 45·4 (see Table 2). The fact that the younger smolts tend to remain longer feeding in the sea than the older smolt ages is illustrated in Table 3.

Although the results of the present investigation with regard to the age of the smolts are of the same order as those obtained previously (Nall, 1931, and Went and Barker, 1943) there is an increase in the proportion of two-year smolts almost entirely at the expense of the three-year smolts, despite the fact that the proportion of fish in their first post-migration summer is much greater than in the previous investigations. Nevertheless these differences are well within the fluctuations which one might normally expect in a river from time to time.

Age at maturity and number of spawning marks.

In the previously-spawned fish, 29·3 per cent. had spawned for the first time in their first post-migration winter, 57·2 per cent. in their second post-

migration winter, and 13·5 per cent. in their third post-migration winter. There is an apparent discrepancy between the results as obtained from the examination of previously-spawned fish (Table 4) and those obtained by

Table 1. Showing the time and place of the collection of the material from the rod catches in 1943 (see Went and Barker, 1943, page 84 of sketch map of catchment area).

Locality from which data obtained	No. of sets of data collected	Month	No. of sets of data collected
Waterville River	34	Up to and including 30th June	155
Lough Currane	715	July	219
Derriana Lough	37	August	192
Loughnamona	6	September	221
Cloonaghlin Lough	27	1st Oct. to 12th Oct. both days inclusive	58
Loughnahiska	3		
Coppal Lough & River	23		
Total	845	Total	845

Table 2. Proportion of the different smolt ages (maiden fish only) as percentages of each age group.

Age Group	Smolt Age				Total
	2 year	3 year	4 year	5 year	
1st Post Migration Summer	45.4	43.9	8.9	1.8	100.0
2nd, 3rd and 4th Post Migration Summers	73.3	25.7	1.0	-	100.0
Total	56.0	37.0	5.9	1.1	100.0

Table 3. Distribution of the various age groups in each smolt class (as percentages)

Age Group	Smolt Age				Total
	2 year	3 year	4 year	5 year	
1st Post Migration Summer	50.0	73.5	93.5	100.0	62.0
2nd, 3rd and 4th Post Migration Summers	50.0	26.5	6.5	-	38.0
Total	100.0	100.0	100.0	100.0	100.0

Table 4. Age at first spawning in the different smolt classes (as percentages)

Age at first spawning	Smolt Age				Total
	2 year	3 year	4 year	5 year	
1st Post Migration Year	21.5	37.4	33.3	100.0	29.3
2nd Post Migration Year	63.3	50.7	61.9	-	57.2
3rd and 4th Post Migration Years	16.2	11.9	4.8	-	13.5
Total	100.0	100.0	100.0	100.0	100.0

Table 5. Number of spawning marks present in previously spawned fish.

	Number of spawning marks					Total
	1 SM	2 SMs	3 SMs	4 SMs	5 SMs	
Weighted Number	403	119	57	31	12	622
Percentage	64.9	19.1	9.1	5.0	1.9	100.0

Table 6. Proportion of previously spawned fish in the different months (weighted figures used)

Month	Maiden Fish	Previously Spawned Fish
Up to 31st March	10%	90%
April	7%	93%
May	37%	63%
June	74%	26%
July	93%	7%
August	86%	14%
September	85%	15%
Total	77%	23%

Table 7. Calculated mean lengths in inches in fresh water.

Smolt Age	No.	Mean length at end of					Mean Smolt Length
		1st Winter	2nd Winter	3rd Winter	4th Winter	5th Winter	
2	388	3.8	9.0	-	-	-	9.7
3	266	3.1	7.2	9.1	-	-	10.1
4	42	2.5	5.9	8.3	10.0	-	10.4
5	5	2.4	5.4	8.3	10.5	11.8	12.1

Table 8. Calculated mean lengths in inches in the sea.

Age Group	No.	Mean Smolt Length	Mean length at end of			Mean length at Capture
			1st Winter	2nd Winter	3rd Winter	
1st Post Migration Summer	430	9.8	-	-	-	12.0
2nd Post Migration Summer	237	10.5	14.1	-	-	16.0
3rd Post Migration Summer	33	10.1	14.0	17.6	-	20.9
4th Post Migration Summer	1	10.5	13.0	15.2	18.1	19.5
Total	701	9.85	14.0	17.6	18.1	-

Table 9. Calculated mean length in inches at the end of each year of life in the river and sea (for maiden fish only).

Group	Number Examined	River Life						Sea Life		
		Mean length at end of					Mean Smolt Length	Mean length at end of		
		1st Winter	2nd Winter	3rd Winter	4th Winter	5th Winter		1st Winter	2nd Winter	3rd Winter
1+	198	3.0	7.4	-	-	-	9.3	-	-	-
2+	189	3.1	7.4	8.9	-	-	10.1	-	-	-
3+	38	2.5	5.8	8.2	9.9	-	10.4	-	-	-
4+	5	2.4	5.4	8.3	10.5	11.8	12.1	-	-	-
1.1+	171	4.1	9.7	-	-	-	10.5	14.4	-	-
2.1+	64	3.1	6.9	9.4	-	-	10.0	13.2	-	-
3.1+	2	3.2	6.0	8.6	10.3	-	10.8	14.3	-	-
1.2+	18	4.2	9.2	-	-	-	10.1	13.3	16.8	-
2.2+	13	3.2	6.9	9.8	-	-	10.0	14.0	17.7	-
3.2+	2	2.8	6.2	9.0	10.3	-	10.3	13.6	16.6	-
2.3+	1	3.1	9.2	-	-	-	10.5	13.0	15.2	18.1

examination of the maiden fish (Table 3). This discrepancy is more apparent than real, because it is well known that large numbers of whitling (i.e. fish in their first post-migration summer) enter fresh water, sometimes remaining there over the winter, and descend again to the sea without spawning, often accompanied by other sea trout as kelts.

If one assumes that the conditions in the Waterville River are accurately represented by the results given in Tables 3 and 4 then out of every 100 fish spawning, approximately 29 entered the river in their first post-migration summer, i.e. the proportion of whitling to other age groups was 29 to 71. In the entrants to the river in the maiden state the ratio of whitling to other fish was 62 to 38 or say 116 to 71. It follows, therefore, that out of every 116 whitling (fish in their first post-migration summer) only 29 or 25 per cent. spawned in the winter following their first return to fresh water.

It was calculated that 64·9 per cent. of the previously-spawned fish had a single spawning mark on each of their scales, 19·1 per cent. had two marks, 9·1 per cent. three marks, 5·0 per cent. four marks, and 1·9 per cent. five spawning marks (Table 5). It will be noticed that the proportion of previously-spawned fish having a large number of spawning marks on their scales is very much less than in the spring-running sea trout (Went and Barker, 1943, p. 94). Only one fish had spent more than a year recovering from spawning.

Proportion of previously-spawned fish.

As mentioned previously it is impossible to tell the date of entry into the river of an individual fish which has been taken by rod and line. Nevertheless in order to obtain some idea of the fluctuations in the proportion of maiden and mature fish over the season any fish taken on rod and line from the 1st to the 15th day of every month (both days inclusive) was included in the catch for the previous month on the grounds that fresh run fish take a lure more readily than fish some time in fresh water. In Table 6 the results have been given, and it will be seen that in the early part of the season, February to May inclusive, the previously-spawned fish predominated, whereas after that time the maiden fish formed three-quarters or more of the total stock.

Condition co-efficient.

The relationship between the weight and length of a fish, usually called the "condition co-efficient" or "condition factor," is a useful means of assessing the differences in shape which occur over the season or in different districts. As Nall explains (Nall, 1930, p. 263) an accurate comparison cannot be made between fish of different rivers unless they are examined in the same year and month, and after the same interval in fresh water. Obviously with rod-caught fish this ideal test is impossible to achieve. Nevertheless the comparisons are of sufficient interest if we bear in mind that we are dealing, for the most part, with fish which have been up in fresh water for some time before capture.

In the case of sea trout the formula used by workers dealing with British units of weight and length is that devised by Menzies, when working on the sea trout of the River Forth and subsequently adopted by Nall in his intensive studies on Scottish sea trout, namely, $K = W/(L^3 \times 0{\cdot}000427)$ where W is the weight in lbs. and L is length in inches. Another factor, usually called the "condition factor" (according to Corbett's scale) is also sometimes used, particularly by anglers and it is calculated according to the formula C.F. $= 10^5 W/L^3$, where W and L have the same meaning as in the formula mentioned previously. The relationship between the two factors is obviously $K \times 42{\cdot}7 =$ C.F.

In Table 10 the mean condition co-efficients for the various groups have been given, and, even taking into consideration the fact that most of the fish examined had been for some time in fresh water before capture, it seems apparent that sea trout of the Waterville River are slim fish, but it is well known that this character has not affected either their sporting or edible qualities. There is, however, no indication that spawning has affected the average condition of the fish.

Calculated lengths.

As mentioned previously (page 202) the length at the end of every year of life of each fish having suitable scales was calculated in the normal way, i.e. on the assumption that the growth of the fish was strictly proportional to the growth of the scale. Normally the fish having spawning marks on their scales are excluded for obvious reasons. In other words, only maiden fish are deemed to have suitable scales for this purpose. In the previous investigation (Went and Barker, 1943), as in Nall's investigation (Nall, 1931), the scales of a large proportion of previously-spawned fish were also measured, and the appropriate lengths calculated therefrom. It was, of course, realised that the results from such measurements were likely to be somewhat inaccurate, but with the limited number of maiden fish available for examination this could not be avoided. In this investigation the maiden fish sampled, over 700 in number, were sufficiently numerous to give reliable results.

(*a*) *River Life.*—In Table 7 the mean calculated lengths for each smolt class at the end of each year of fresh-water life have been given, and the growth and frequency distribution curves have been given in Fig. 2. It will be seen that the fastest growing parr migrated first.

In the previous work on sea trout of this river (Went and Barker, 1943) it was mentioned that a large proportion of the parr of these fish showed on their scales certain growth rings which correspond with a period of rapid growth in fresh water in the spring prior to migration as a smolt. The transition from river to sea growth is quite well marked in these fish. The difficulty experienced elsewhere with regard to estuarine growth, i.e. growth in the brackish waters of the estuary, is absent, presumably because the Waterville River flows directly into Ballinaskelligs Bay, which opens direct

into the Atlantic Ocean, and in consequence there is no brackish water to provide feeding grounds for the sea trout. It is, therefore, quite simple to calculate the length of the smolts from the scales, and this was done in the

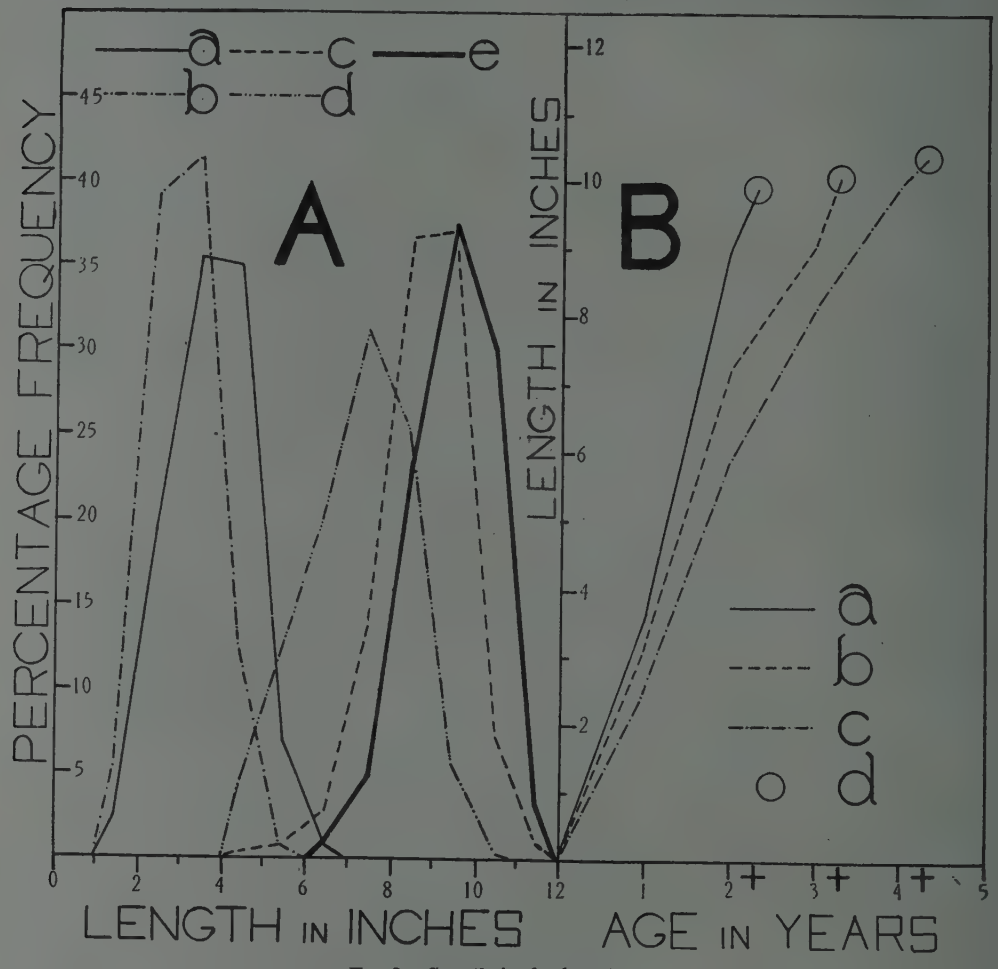


FIG 2.—Growth in fresh water.

A. Distribution curves—
- (*a*) Two-year smolts at one year.
- (*b*) Three-year smolts at one year.
- (*c*) Two-year smolts at two years.
- (*d*) Three-year smolts at two years.
- (*e*) Three-year smolts at three years.

B. Growth curves in the different smolt classes—
- (*a*) Two-year smolt class.
- (*b*) Three-year smolt class.
- (*c*) Four-year smolt class.
- (*d*) Size at migration.

majority of cases. The proportion of fish, which did not show fresh-water growth in the spring prior to migration to the sea as smolts, was exceedingly small, and there was, therefore, no point in separating the fish into two types, as was done in the case of salmon of a number of Irish rivers (see Went, 1938 to 1943). The mean length of the smolt in the various smolt classes increased with a rise in the smolt age (Table 7).

From Table 9 it is obvious that the differentiation in freshwater growth in the various age groups of any one particular smolt class is slight, although the older age groups, i.e. fish in their second and third post-migration summers, have, on the average, a slightly more rapid growth rate in fresh water than fish in their first post-migration summer.

(*b*) *Sea Life.*—Although the number of observations of maiden fish had increased twelvefold, the single specimen of a fish, which returned to fresh water in its fourth post-migration summer described previously (Went and Barker, 1943, page 95) was the only member of its group to be noted in this series of investigations. The calculated lengths at the end of each winter of sea life have been given in Table 8, and it will be seen that in the fish in their second and third post-migration summers the mean lengths at the end of the first sea winter are almost identical. The actual increments made in the sea up to the end of the first sea winter were 3·6 inches and 3·9 inches, respectively, for fish in their second and third post-migration summers (see Table 8), the mean growth rate for all fish being 3·6 inches.

Table 10. Average sizes in the different age groups.

Age Group	Number examined	Length in inches			Mean weight in lb.	Mean condition coefficient K	Mean condition factor C.F.
		Minimum	Maximum	Mean			
.+	437	9.0	15.5	12.0	0.67	0.90	38.4
.+ 1 SM+	50	13.5	19.75	14.9	1.25	0.82	35.0
.+ 2 SM+	10	15.5	25.0	18.7	2.70	0.86	36.7
.+ 3 SM+	9	17.25	25.0	21.2	4.82	0.90	38.4
.+ 4 SM+	6	19.5	22.8	21.8	4.93	0.87	37.1
.+ 5 SM+	3	20.9	23.2	22.1	6.25	0.90	38.4
.1+	272	11.0	19.25	16.3	1.49	0.84	35.8
.1+ SM+	121	13.5	24.5	19.3	2.54	0.85	36.7
.1+ 2 SM+	73	18.9	25.0	21.7	3.80	0.90	38.4
.1+ 3 SM+	52	18.2	25.5	22.7	4.42	0.94	40.1
.1+ 4 SM+	23	20.9	25.0	23.4	4.78	0.89	38.0
.1+ 5 SM+	6	23.0	26.0	24.6	5.50	0.83	35.4
.1+ SM .1+	1	-	-	24.0	5.75	0.98	41.8
.2+	34	15.75	24.0	20.9	3.96	0.89	38.0
.2+ SM+	41	16.25	25.0	21.7	4.03	0.88	37.6
.2+ 2 SM+	15	21.0	25.5	22.6	4.33	0.85	36.7
2+ 3 SM+	12※	20.5	30.0	26.5	6.34	0.95	40.9
2+ 5 SM+	3	25.0	27.0	25.7	5.75	0.80	34.2
3+	1	-	-	19.5	-	-	-
Total	1169	9.0	30.0	-	-	0.88	37.6

※ Including the largest specimen examined 30 inches long and 10 lb. in weight.

Table 11. Size Distribution (Percentage of weighted numbers). See also Fig. 3.

Age Group	Length in inches ※																			Total
	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	30	
1st Post Migration Summer	1.1	5.5	12.8	18.9	6.4	0.8	0.2	-	0.1	-	-	-	-	-	-	-	-	-	-	-	45.8
2nd Post Migration Summer	-	-	0.4	0.5	1.2	3.9	7.5	9.1	3.8	2.1	0.7	0.2	-	-	-	-	-	-	-	-	29.4
Total	1.1	5.5	13.3	19.4	8.6	6.7	9.7	10.6	6.1	3.8	2.4	3.3	2.9	2.5	2.1	1.2	0.5	0.1	0.1	0.1	100.0

※ Class interval 9 etc. includes all fish having lengths between $8\frac{7}{8}$ and $9\frac{7}{8}$ inches etc.

Average sizes.

The average sizes of the various groups of fish have been given in Table 10. In general it might be said that there is an increase in the mean length and weight after each recovery from spawning, but the annual increment made after the initial spawning is somewhat less than that in the maiden fish. This is not unexpected, as apart from the fact that a kelt has to recover the weight lost as a result of spawning activities, the period of feeding in the sea in any one year is less than in the maiden fish.

Oldest and heaviest fish.

Two fish (0·2 per cent.) were in their eleventh year since hatching, five (0·4 per cent.) in their tenth year, thirty-one (2·6 per cent.) in their ninth year, and sixty-six (5·6 per cent.) in their eighth year. It will be noted that the proportion of old fish is less than that given in the earlier paper, owing to the inclusion of large numbers of late-running sea trout, a large part of which were maiden or immature fish and, therefore, relatively young. The

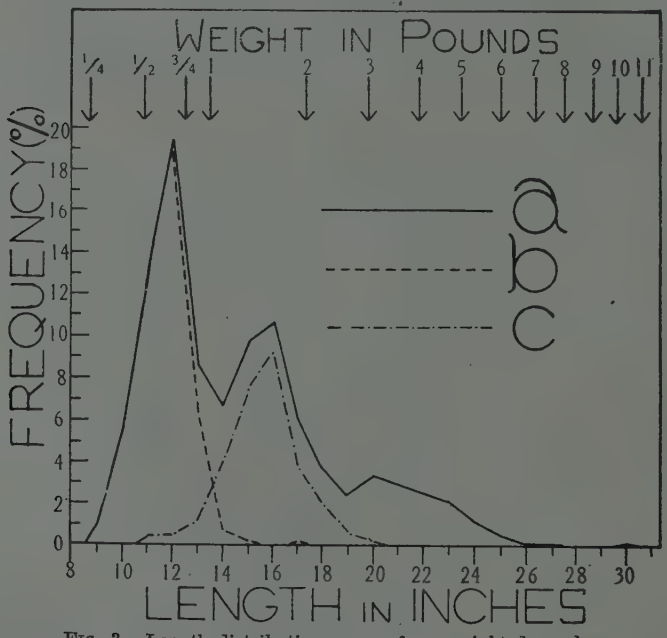


FIG. 3.—Length distribution curves from weighted numbers.

(*a*) All fish.
(*b*) Whitling (maiden fish in their first post-migration summer).
(*c*) Maiden fish in their second post-migration summer.

(NOTE.—The weight scale drawn up on the assumption that the mean condition coefficient (K) = 0·9.)

heaviest fish was one referred to previously (Went and Barker, 1943, page 96) weighing 10 lbs. and measuring 30 inches.

Size distribution.

The size distribution of the sea trout was estimated from the available data, and the results have been given in Table 11 (see also Fig. 3). It will be seen that there are two peaks in the curve corresponding to the individual curves for the maiden fish in their first and second post-migration summers. In order that the angler should be able to assess the proportion of the various sizes, on the basis of the weight, a scale has been added to Fig. 3 for this purpose, the weight being determined on the assumption that the average condition coefficient was 0·9.

Discussion of results.

In Went and Barker, 1943, p. 97, it was suggested that examination of the late-running sea trout might have given somewhat different results from those obtained by consideration of the spring-running fish. It is now clear, however, that such was not the case when one considers the growth of and size attained by the smolts, and the spring-running fish are obviously merely part of the general stocks of sea trout in this river; not a separate population or entity. The fact that sea trout are to be found in the fresh condition in the early part of the season in the Waterville River is due, in the main, to the early return of the previously-spawned fish. This cannot be explained at present. It is not on account of the necessity to penetrate great distances to the spawning beds, because even to reach the uppermost waters of this river system trout have comparatively short distances to travel.

By combining the material from the net and weir captures with that from the rods a reasonably accurate idea of the stocks of sea trout in the Waterville River can be obtained. The rapid growth in fresh water and the large size attained by the smolts are, more or less, unique as far as sea trout have been investigated to date.

Résumé.

(1) New material, consisting of sets of scales and data from sea trout taken by single rod and line in the Waterville River system was obtained and subjected to a preliminary examination. As there was no sign, based on the estimated length of the smolts, of a division of the maiden fish and those maiden fish examined previously (Went and Barker, 1943) into two or more distinct populations (Fig. 1) the new material was added to the material examined previously and the combined material was used thereafter. The possibility of anglers taking quite unwittingly the fry of sea trout has been discussed.

(2) The bulk of the fish migrated as two and three-year smolts the latter being only two-thirds as numerous as the former. There was a tendency for the younger smolts (2 years old) to return to fresh water after a longer interval in the sea than the other smolt classes (Tables 2 and 3).

(3) Just over three-quarters of the total run were estimated to have been maiden fish, and of these nearly 60 per cent. were in their first post-migration summer. As the season progressed the proportion of previously spawned fish, which at first predominated, gradually decreased, and towards the end of the season (from July onwards) less than one-sixth of the total had spawned before (Table 6).

(4) Only 25 per cent. of the fish returning to the river for the first time in their first post-migration summer spawned in the following winter. The remainder may have wintered in fresh water, but did not spawn until, at least, the succeeding winter (Tables 3 and 4).

(5) Previously spawned fish had from one to five spawning marks on their scales. The fish which had only one such mark amounting to about two-thirds of the total, those having two spawning marks were less than one-third as abundant (Table 5).

(6) The lengths at the end of each year of life were calculated for all maiden fish having suitable scales. The fastest growing fish migrated first and there was a rise in average length of the smolts as the smolt classes were ascended (Table 7 and Fig. 2). There appeared to be a slight differentiation in size attained in fresh water in the different age groups (Table 9 and Fig. 1). At the end of the first winter in the sea there was very little difference in mean length in the different age groups (Table 8).

(7) The average sizes in the various groups have been given in Table 10. After each successive spawning the mean length increased, but the increment gradually became smaller (Table 10).

(8) Even allowing for the fact that some fish had been some time in fresh water before capture the average condition coefficient (0·88) suggests that the sea trout of this river are slim fish (Table 10).

(9) In Table 11 the estimated size distribution of the sea trout runs have been given (see also Fig. 3).

ACKNOWLEDGMENTS.

I wish to take this opportunity of expressing my thanks to Mr. G. P. Farran, B.A., Chief Inspector of Fisheries, Department of Agriculture, Dublin, for his interest in the progress of the work and for his many helpful suggestions as regards presentation of the material. My grateful thanks are also due to Mr. P. J. Hamilton, Clerk and Inspector to the Waterville Board of Fishery Conservators, for collecting the material and to the numerous visiting anglers to Waterville for their kindness in allowing their catches to be sampled.

REFERENCES.

(1) Department of Agriculture, Fisheries Branch, "Abstract of Fishery By-laws," 1937 Edition, Dublin.
(2) NALL, G. H.—1929. "Irish Sea Trout, Notes on a small collection of sea trout scales from the Louisburg District of County Mayo." Salmon and Trout Magazine, No. 55, April, 1929.
———1930. "The Life of the Sea Trout," London.
———1931. "Irish Sea Trout; Notes on collections of scales from the West Coast of Ireland," Proc. Roy. Irish Acad., **40**, B, No. 1.
(3) Statute—"5 and 6 Vic. Cap. 106, Fisheries (Ireland) Act, 1842."
(4) WENT, ARTHUR E. J.—1938. "Salmon of the River Shannon," Proc. Roy. Irish Acad., **44**, B, No. 11.
———1940. "Salmon of the River Shannon," Journal of the Department of Agriculture, **37**, No. 2, Dublin, 1940.
———1941. "Salmon of the Ballisodare River II, Age and Growth," Sci. Proc. R. Dublin Soc., **22** (N.S.), No. 35.
———1941. "Salmon of the Owenduff (Ballycroy) River," Proc. Roy. Irish Acad., **47**, B, No. 6.
———1942. "Salmon of the River Erne. Results of the examination of a small collection of scales and data." Sci. Proc. R. Dublin Soc., **22** (N.S.), No. 49.
———1943. "Salmon of the River Corrib," Proc. Roy. Irish Acad., **48**, B, No. 12.
———1943. "Salmon of the River Shannon," Proc. Roy. Irish Acad., **49**, B, No. 9.
(5) WENT, ARTHUR E. J., and T. S. BARKER.—1943. "Salmon and Sea Trout of the Waterville (Currane) River," Sci. Proc. R. Dublin Soc., **23**, (N.S.), No. 9.

No. 21.

REACTION OF *p*-DIMETHYLAMINOBENZALDEHYDE WITH AROMATIC AMINO COMPOUNDS.

By A. E. A. WERNER.

[Read March 28. Published separately April 28, 1944.]

Weil (1) has pointed out that *p*-dimethylaminobenzaldehyde reacts with aromatic amines to give intense orange precipitates, which he examined in a qualitative way. Sachs and Lewin (2) and Möhlau (3), in discussing the colour of azomethine compounds, refer briefly to compounds which *p*-dimethylaminobenzaldehyde forms with aniline, *p*-toluidine, and *p*-aminodimethylaniline. The present author has described a method for the detection and quantitative estimation of sulphanilamide (4) and sulphapyridine (5) which depends on the formation of an intense yellow colour (or in concentrated solutions an orange precipitate) upon the addition of an acid solution of *p*-dimethylaminobenzaldehyde. Tauber and Laufer (6) have also described a similar colour test for *p*-aminobenzoic acid, depending upon the formation of an orange colour with *p*-dimethylaminobenzaldehyde in glacial acetic acid solution.

In the present communication a systematic investigation of the reaction of *p*-dimethylaminobenzaldehyde with over fifty different amino compounds has been undertaken. The qualitative results obtained may be conveniently summarised thus:—

(*a*) Aromatic compounds react in the presence of mineral acid, provided the $-NH_2$ group is attached directly to the benzene nucleus. Thus aniline reacts, but not benzylamine.

(*b*) Compounds in which a nuclear carboxylic or sulphonic group are present, e.g. *p*-aminobenzoic acid or sulphanilic acid, react immediately in the absence of mineral acid.

(*c*) No reaction occurs with (i) aliphatic amines and amino acids, (ii) N-substituted aromatic amines, (iii) heterocyclic amino compounds, or (iv) amino derivatives of the cycloparaffins, e.g. cyclohexylamine.

The fact that such compounds as methylaniline and acetanilide give no colour when treated with *p*-dimethylaminobenzaldehyde supports the hypothesis suggested by the author and Morris (7), that the chromophoric complex responsible for colour formation is of the Schiff base type, being formed by elimination of water between the aldehydic group and the primary amino group of the aromatic amine. This hypothesis, however, fails to account for the fact that, whereas the Schiff bases normally are feebly coloured substances, the chromophoric complex in question has a much more intense colour; there must be some other factor of paramount importance, which it is the main purpose of the present communication to investigate. With this end in view the

reaction of *p*-dimethylaminobenzaldehyde with sulphanilamide, aniline, and *p*-aminobenzoic acid has been investigated in greater detail.

When equimolecular amounts of *p*-dimethylaminobenzaldehyde and sulphanilamide were suspended in water, no trace of colour developed, but the addition of a strong mineral acid produced an immediate orange precipitate, a maximum yield being obtained when exactly one equimolecular proportion of acid had been added. This orange precipitate proved to be a salt of the Schiff base with the added acid, so that the reaction may be adequately represented by the following equation:—

$$Me_2N-C_6H_4-CHO + H_2N-C_6H_4-SO_2-NH_2 + HX = [Me_2N-C_6H_4-CH=N-C_6H_4-SO_2-NH_2]HX + H_2O$$

The above constitution was proved by analysis of the hydrochloride salt, which was found to contain two molecules of water of crystallisation. This *orange* salt is readily converted by the addition of an equimolecular proportion of alkali or ammonia into a *pale-yellow* compound, which proved to be identical with the Schiff base obtained by Gray, *et al.* (8) on fusing sulphanilamide with *p*-dimethylaminobenzaldehyde. Conversely, the Schiff base so obtained was readily converted into the orange-coloured salt upon the addition of acid. The orange salt obtained by adding *p*-dimethylaminobenzaldehyde to a concentrated solution of aniline hydrochloride behaved in a similar way, and was converted by the addition of alkali or ammonia into an insoluble Schiff base identical with that obtained by Sachs and Lewin (*loc. cit.*) by heating a mixture of aniline and *p*-dimethylaminobenzaldehyde.

This reversible interconversion may be represented thus:—

$$\underset{\text{Orange}}{[Me_2N-C_6H_4-CH=N-C_6H_5]HX} \underset{\text{acid}}{\overset{\text{alkali}}{\rightleftharpoons}} \underset{\text{Pale-Yellow}}{Me_2N-C_6H_4-CH=N-C_6H_5}$$

The insoluble orange "zwitterionic" salt obtained from *p*-aminobenzoic acid, if suspended in water and treated with alkali, goes into solution with a pale-yellow colour, containing the Schiff base,

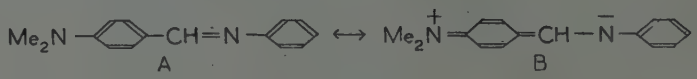
$$Me_2N-C_6H_4-CH = N-C_6H_4-COONa.$$

On standing the pale-yellow colour slowly faded, and glistening crystals separated out, which proved to be *p*-dimethylaminobenzaldehyde, produced by the slow hydrolysis of the Schiff base according to the equation:—

$$Me_2N-C_6H_4-CH=N-C_6H_4-COONa + H_2O = Me_2N-C_6H_4-CHO + H_2N-C_6H_4-COONa$$

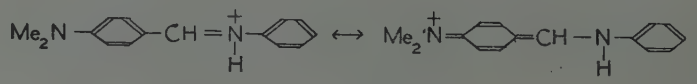
In order to understand the marked difference in the intensity of colour between the *orange* salt and the *pale-yellow* free base, it is necessary to consider the intimate molecular structure of the kation of the salt in the light of two factors, which are now recognised as being of paramount importance in connection with the development of colour in organic compounds. These two factors are (i) a conjugated double bond system (cf. Burawoy, 9), and (ii) a resonating system (cf. Brooker *et al.*, 10).

Applying these considerations to the simplest example, namely, the compound formed between *p*-dimethylaminobenzaldehyde and aniline, it is to be noted that the free base can give rise to a resonating system to which the components (A) and (B) contribute :—



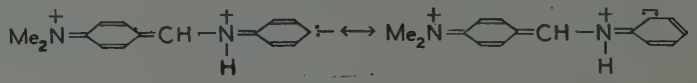
A B

The component (B), however, is much less stable than (A), due to the presence of the negatively charged nitrogen atom, and also the quinonoid ring system[1], and therefore the amount of resonance is limited. Hence the free base has a light colour, due to the presence of the conjugated double bond system in (A). Upon acidification, however, a proton readily adds itself at the negatively charged nitrogen atom, giving the kation of a salt, in which unrestricted resonance is possible. A resonating system results, in which the components (C) and (D)



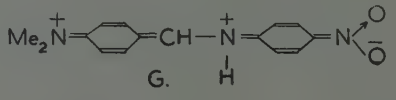
C. D

are of primary importance, but to which the components (E) and (F) also contribute :—

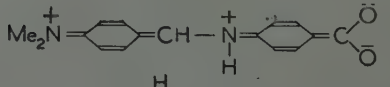


E. F.

The extent to which the components (E) and (F) contribute to the resonating system will be increased, if suitable electron accepting substituents are present in the *ortho* and *para* positions in the aromatic nucleus of the reacting amino compound; thus, in the case of *p*-nitraniline or *p*-aminobenzoic acid, components of the type (G) and (H) will contribute largely to the resonating system.



G.



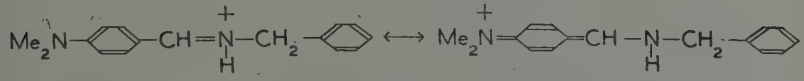
H.

[1] The possibility of resonance between the different Kekulé and other configurations of the benzene ring has a stabilising effect on the benzenoid configuration, which is therefore strongly favoured over the quinonoid configuration,

Furthermore, the more such components contribute to the resonating system, the more perfect the resonance becomes, and hence the more intense the colour of the kation. This deduction has been verified, at least qualitatively, by the experimental findings that the colour given by such aromatic amines as aniline or toluidine is relatively weak in comparison with the colour given by *p*-nitraniline, *p*-aminobenzoic acid, or sulphanilic acid. A quantitative investigation of this aspect of the exact relationship between intensity of colour and chemical structure of the reacting amino compounds has been postponed to a later communication. Passing reference may be made to the fact that the presence of bulky substituents in the *ortho* position to the $-NH_2$ group apparently suppresses the colour intensity. Thus *o*-nitraniline and *sym*-tribromaniline fail to develop any colour upon the addition of *p*-dimethylaminobenzaldehyde.

The essential correctness of the above hypothesis concerning the structure of the kation and the development of colour in these compounds receives additional support from a consideration of the following points:—

(*a*) Aliphatic amino compounds, benzylamine or cyclohexylamine, give no colour upon acidification, although a Schiff base can be prepared according to the fusion method. Now the kation of such compounds can only exhibit resonance to a very limited extent; structures such as (E) and (F) are clearly impossible, and although resonance between components of the type (C) and (D) may be theoretically visualised, e.g. for benzylamine:—



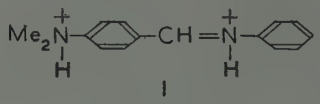
this resonance will be severely damped, if not entirely absent, since the positive charge of the kation must be almost entirely localised on the more basic nitrogen atom. An approximately quantitative idea of the N-N imbalance may be obtained by comparing the ionisation constant of dimethylaniline, namely, $2{\cdot}4 \times 10^{-10}$, with that of benzylamine, namely, $2{\cdot}5 \times 10^{-5}$. (Cyclohexylamine is such a strong base that its aqueous solution readily absorbs carbon dioxide.) Since resonance in the kation is almost entirely suppressed, no colour is developed. There is not even the feeble yellow colour associated with a Schiff base, since "perfect conjugation" (cf. Burawoy, *loc. cit.*) throughout the molecule is absent, conjugation being confined to the three double bonds in the aldehydic portion of the molecule.

(*b*) The colour of the kation depends upon the hydrogen ion concentration. In *dilute* solution an equilibrium is established which may be represented by the following equation:—

$$Me_2N-C_6H_4-CH=N-C_6H_5 + HX \rightleftharpoons [Me_2N-C_6H_4-CH=\overset{+}{\underset{\underset{H}{|}}{N}}-C_6H_5]\,X^-$$

On addition of a mineral acid to an equimolecular mixture of *p*-dimethylaminobenzaldehyde and an aromatic amine there is at first a rapid increase

in colour intensity, which attains a maximum (see experimental part) and then falls off gradually until the solution is finally colourless. The initial increase in colour intensity is due to a shift in the equilibrium in favour of the kation concentration; further addition of acid causes the formation of a doubly charged kation of the structure (I)



I

by the addition of a second proton. A kation of this type is not capable of existing in a resonating state, and is therefore colourless.

(*c*) The colour of the kation is sensitive to the action of heat. On heating an aqueous solution of the kation, the colour intensity gradually diminishes as the temperature increases. This may be attributed to the normal thermal dissociation that all ammonium salts undergo, and in this case involves the following change:

$$[Me_2N-C_6H_4-CH=\overset{+}{\underset{|}{N}}\underset{H}{}-C_6H_5]\,X^- \rightleftharpoons Me_2N-C_6H_4-CH=N-C_6H_5 + HX$$

leading to the formation of the feebly coloured Schiff base. On cooling the solution, the colour returns to its original intensity. A similar colour change is also observed on heating the solid salt.

(*d*) Addition of sodium chloride in large excess causes an increase in the colour intensity, whereas addition of ethyl alcohol causes a marked decrease in the colour intensity. Both these salt and solvent effects are associated with a substance in which the following equilibrium with an acid is established:—

$$B + HX \rightleftharpoons B^+H + X^- .$$

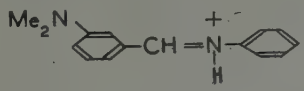
The law of mass action takes the form (11)

$$\frac{[B^+H][X^-]}{[B][HX]} = K\frac{f_B \cdot f_{HX}}{f_{B^+H} \cdot f_{X^-}}$$

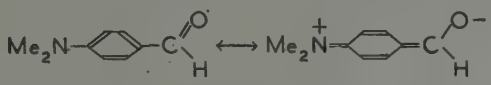
so that the addition of an inert electrolyte, e.g. NaCl, would cause a shift of the equilibrium in favour of the kation B^+H, since it decreases the ionic activity coefficients f_{B^+H} and f_{X^-}. Conversely, the replacement of water by alcohol decreases f_{B^+H} and f_{X^-} thus causing the equilibrium to shift in favour of the free base B, with consequent decrease in colour intensity. It must be noted, however, that the above remarks do not apply to those salts in which sulphonic or carboxylic groups are present in the molecule; in such cases the salt and solvent effects are not so definite nor predictable on theoretical grounds.

(*e*) No colour is obtained when *m*-dimethylaminobenzaldehyde is used in place of the *para* isomeride. There was evidence of Schiff base formation, when the components were heated together on a water-bath, but addition of

a mineral acid did not produce any intense coloration. This lack of colour in the kation having the structure



must be attributed to the fact that resonance is impossible. Furthermore, the fact that Schiff base formation does not readily occur is further evidence of the decreased activity of the carbonyl group in *m*-dimethylaminobenzaldehyde, a fact which has been experimentally shown by Cocker, *et al.* (12), in other reactions of this compound. This reduced reactivity of the carbonyl group is probably due to the fact that, whereas the *meta* isomeride cannot resonate, the *p*-dimethylaminobenzaldehyde itself does show resonance between the structures



EXPERIMENTAL.

Condensation of p-dimethylaminobenzaldehyde with sulphanilamide.—(*a*) Finely powdered *p*-dimethylaminobenzaldehyde (7·5 g.) was intimately mixed with finely powdered sulphanilamide (8·6 g.), and dissolved in hot distilled water (1,000 c.c.). Upon addition of N hydrochloric acid (50 c.c.), the hydrochloride of the Schiff base separated as a copious orange precipitate. Yield = 13·3 g., corresponding to 85 per cent. of the theoretical. Recrystallised from hot water acidulated with hydrochloric acid in the form of dark-red acicular crystals. M.P. 242° C. Analysis (after drying at 105° C.) Found C = 53·20 per cent.; H = 5·31 per cent.; N = 12·38 per cent.; S = 9·55 per cent.; HCl = 10·65 per cent. (argentometrically), 10·70 per cent. (titrimetrically) with N/10NaOH); $C_{15}H_{17}O_2N_3S$. HCl requires C = 53·0 per cent.; H = 5·30 per cent.; N = 12·36 per cent.; S = 9·40 per cent.; HCl = 10·75 per cent. Loss of weight on drying at 105° C. = 9·4 per cent. $C_{15}H_{17}O_2N_3S$. HCl . $2H_2O$ requires 9·6 per cent. By using other mineral acids the following salts were obtained:—Sulphate, M.P. 212° C.; Nitrate, M.P. 200° C.; Oxalate, M.P. 195° C.; Picrate, M.P. 187° C.

(*b*) *p*-dimethylaminobenzaldehyde (7·5 g.) and sulphanilamide (8·6 g.) were dissolved in hot distilled water (1,000 c.c.). Addition of N acetic acid (50 c.c.) caused gradual formation of a lemon yellow precipitate. Yield = 13·0 g., 81 per cent. of the theoretical, M.P. 214° C. (Found N = 13·80 per cent. $C_{15}H_{17}O_2N_3S$ requires N = 13·90 per cent.)

(*c*) *p*-dimethylaminobenzaldehyde (3·8 g.) and sulphanilamide (4·3 g.) were intimately mixed and heated in an oil bath to 140° C. A homogeneous melt is obtained, from which a crystalline solid starts to separate. The solid

mass was maintained at 140° C. for 30 min. The solid residue was extracted with mixture of acetone (20 c.c.) and ethyl alcohol (80 c.c.). Lemon yellow substance remained. Yield = 4·0 g., 54 per cent. of the theoretical, M.P. 212° C. Found N = 13·85 per cent.; S = 10·56 per cent. $C_{15}H_{17}O_2N_3S$ requires N = 13·90 per cent.; S = 10·60 per cent. (Gray *et al., loc. cit.*, give M.P. 229° C.) This compound shows no depression of M.P. when mixed with the compound obtained in (*b*), which must also be the free Schiff base; the expected acetate salt is evidently completely hydrolysed, so that reaction (*b*) provides a better method for obtaining the free base.

Condensation of p-dimethylaminobenzaldehyde with (*i*) *p-aminobenzoic acid,* (*ii*) *anthranilic acid, and* (*iii*) *sulphanilic acid.*—In each case equimolecular proportions of *p*-dimethylaminobenzaldehyde and the respective acids were mixed in distilled water. Rapid deposition of an orange precipitate resulted, and the following compounds were obtained in yields 80–85 per cent. of the theoretical.

(i) Recrystallised from hot water in form of dark-red rosettes. M.P. 245° C. (Found N = 10·23 per cent. $C_{16}H_{16}O_2N_2$ requires N = 10·45 per cent.)

(ii) Recrystallised from hot water as red acicular crystals. M.P. 185° C. (Found N = 10·30 per cent.)

(iii) M.P. 300° C. (Found N = 9·15 per cent. $C_{15}H_{16}O_3N_2S$ requires 9·21 per cent.)

In the case of *p*-aminobenzoic acid the effect of carrying out the condensation in non-aqueous solvents was investigated. Thus in ethyl alcohol and benzene the reaction proceeds very slowly and the yields are much poorer.

Condensation of p-dimethylaminobenzaldehyde with aniline.—(*a*) Finely-powdered *p*-dimethylaminobenzaldehyde (7·5 g.) was suspended in distilled water (400 c.c.) and treated with a solution of aniline (5 c.c.) in N hydrochloric acid (50 c.c.). The solution assumed a yellow colour and rapidly deposited an orange precipitate, which on recrystallisation from N/2 hydrochloric acid yielded red acicular crystals. Yield = 11·5 g., 88 per cent. of the theoretical M.P. 216° C. Found N = 10·65 per cent.; HCl = 13·60 per cent. $C_{15}H_{17}N_2Cl$ requires = 10·71 per cent.; HCl = 13·63 per cent..

(*b*) An intimate mixture of *p*-dimethylaminobenzaldehyde (3·00 g.) and aniline (2 c.c.) was maintained at 120° C. for 30 min. The solid residue was dissolved in hot ethyl alcohol (50 c.c.), from which pale yellow crystals were deposited. M.P. = 99·5° C. Found N = 12·35 per cent. $C_{15}H_{16}N_2$ requires N = 12·50 per cent. Sachs and Lewin (*loc. cit.*) give M.P. 100° C.

Conversion of the salts into the corresponding Schiff bases.—(*a*) The finely-powdered hydrochloride salt derived from sulphanilamide (1·5 g.) was suspended in water and treated with a solution (10 per cent.) of ammonium hydroxide. A pale lemon yellow precipitate was deposited from a colourless solution. Yield = 1·0 g. M.P. 212° C. No depression of M.P. observed when mixed with a specimen of *p*-dimethylaminobenzylidene sulphanilamide prepared by fusion method.

(*b*) On treating the same salt (1·0 g.) suspended in water (100 c.c.) with

N/10 sodium hydroxide (31·5 c.c.) a similar pale yellow precipitate was obtained. Yield = 0·55 g. M.P. 212°–214° C.

(*c*) The hydrochloride salt of *p*-dimethylaminobenzylidene aniline (1·5 g.) was suspended in water and treated with a solution (10 per cent.) of ammonium hydroxide. A lemon yellow precipitate was deposited. Yield = 1·2 g. M.P. 96° C. No depression of M.P. on mixing with a specimen of *p*-dimethylaminobenzylidene aniline prepared by fusion method.

Titration with Caustic Soda.—The following experiment is a typical example of the technique used. Millimolecular proportions of *p*-dimethylaminobenzaldehyde and *p*-aminobenzoic acid were dissolved separately in distilled water (500 c.c.). Aliquot portions (50 c.c.) were mixed; the resulting yellow solution was titrated with N/10 sodium hydroxide, and the number of c.c. required to cause complete decolorisation was noted, a sharp end point being easily obtained. Titre obtained = 10·00 c.c., 9·90 c.c. Theory requires 10·00. c.c., corresponding to the equation:—

$$Me_2N-C_6H_4-CH=\overset{+}{N}H-C_6H_4-CO\bar{O} + NaOH = H_2O + Me_2N-C_6H_4-CH=N-C_6H_4-COONa$$

Action of Alkali on p-dimethylaminobenzylidene–p-aminobenzoate.—The compound (0·50 g.) was suspended in distilled water (100 c.c.) and treated with N/10 sodium hydroxide (25 c.c.). A yellow solution resulted. On standing 24 hrs. the solution became colourless. Gradual deposition of crystals ensued. Yield = 0·15 g. M.P. 72° C. Found N = 9·25 per cent. $C_9H_{11}NO$ requires 9·40 per cent. There was no depression of M.P. on mixing with an authentic specimen of *p*-dimethylaminobenzaldehyde.

The Effect of pH on Intensity of Colour.—A centimolecular mixture of *p*-dimethylaminobenzaldehyde and sulphanilamide was dissolved in water (1,000 c.c.), and 1 c.c. portions were added to a series of buffered solutions (5 c.c.), ranging in pH from 1·17 to 4·96. The intensity of colour produced in each case was measured visually in a comparator tube. The colour intensity increased gradually up to a maximum at pH 2·05 and then fell off sharply.

REFERENCES.

1. WEIL.—Ber., **27**, 3317 (1894).
2. SACHS and LEWIN.—Ber., **35**, 3569 (1902).
3. MÖHLAU.—Ber., **31**, 2250 (1898).
4. WERNER.—Lancet, **1**, 18 (1939).
5. WERNER.—Irish Journal of Medical Science, Oct. (1941).
6. TAUBER and LAUFER.—J. Am. Chem. Soc., **63**, 1489 (1941).
7. MORRIS.—Biochem. J., **35**, 952 (1941).
8. GRAY *et al.*—Biochem. J., **31**, 724 (1937).
9. BURAWOY.—J. Chem. Soc., **135**, 1177 (1939).
10. BROOKER, SPRAGUE *et al.*—J. Am. Chem. Soc., **62**, 1116 (1940).
11. BRØNSTED.—J. Chem. Soc., **116**, 574 (1921).
12. COCKER *et al.*—J. Chem. Soc., **134**, 751 (1938).

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THE GAMETOPHYTES OF *PODOCARPUS ANDINUS*.

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(Plates 6 to 9.)

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The simpler species of *Podocarpus* are probably those included in the sub-genus *Stachycarpus*, and of these *P. andinus* is commonly recognized as one of the simplest. There are a number of articles (Coker 1902, Stiles 1912, Sinnott 1913, Buchholz 1941, Tahara 1941) dealing with various developmental phases in the other sections of the genus, notably the Eupodocarpus group. As far as the *pre*-embryonic stages are concerned these papers still leave many points obscure. No complete close study has yet been made of any one species, although in view of the wide differences existing between the various sections it would be desirable to have details, as full as possible, from at least a couple of species from each section for comparison with the anatomical data which are plentifully available. It is still not easy to suggest with a feeling of real probability lines of development within the genus, or fully to distinguish specific and sectional from generic and family characters. For the moment, however, the point of most direct interest here is the fact that there seem to be only two communications referring to *Stachycarpus*. A recent one by Buchholz (1936) deals adequately with the embryogeny proper, but only from the late proembryo onwards, in *P. spicatus*, *P. ferrugineus*, and *P. usumbarensis*. The other (Sinnott 1913) gives an account of earlier stages in development in *P. spicatus* and *P. ferrugineus*, but the account is in general outline only, details of many of the critical phases being lacking or obscure. Thus on the general development in *Stachycarpus* up to embryogeny there is one short incomplete account, now 30 years old, and in which no reference is made to the most interesting species—*P. andinus*. The only direct reference anywhere found to *P. andinus* is contained in a few comments by Stiles (1912) on its pollen grain while still in the pollen sac. When therefore a specimen of this species with a high degree of fertility became available opportunity was taken to make a close study of as many phases as possible in its life-history.

The present paper deals with gametophyte development up to fertilization. In a second paper it is hoped to cover fertilization and embryogeny stages. Both accounts include some items of detail which may later prove to be of little fundamental importance, but until *Acmopyle* and at least some species of *Dacrydium* have been worked out fully—and in both genera primitive features are to be expected—it seemed better to include these items, as they may help later if only to distinguish characters of less importance from those of greater. A few points also overlap some already briefly described by

Sinnott but, as the overlapping is slight, it was again thought better in the interests of clarity to present a continuous account.

There was available at the same time a fertile specimen of *P. nivalis* whose study yielded some new points in development in a species from the Eupodocarpus group. It is hoped to deal with these in another paper, together with some observations on the podocarps as a whole.

MATERIAL.

Specimens of *P. andinus* are to be found in many estates, but, being ordinarily dioecious, fertile ovules and fruit are comparatively rare. The material used for this study was growing in the National Botanic Gardens, Glasnevin, Dublin. One specimen was noticed to develop young ovulate strobili rather freely, but limited to two large branches on one side. Examination showed that those two branches, at some period when the plant was younger, had been tied with wire for support. The marks of the wire and the swellings above the parts to which the wire had been attached were quite obvious, and this disturbance of conduction was clearly associated with the fertility. About 100 yards away, but with a dense cluster of other trees and shrubs between, grew a staminate plant occasionally bearing a little pollen. This however was ineffective for natural pollination, the ovulate strobili fading and falling off without further development as winter set in. A few attempts at artificial pollination showed however that, if pollinated, the ovules could develop further. On the occasion of a visit in 1938 to the gardens of the Hon. Miss Ross at Rostrevor branches were brought back from a specimen there carrying pollen profusely. Surprisingly successful pollination was obtained with this material scattered over the Glasnevin plant, and from the ovules which overwintered and resumed their development in the spring of 1939 the dates of some of the critical stages were approximately determined. By an accident of extreme good fortune the male tree at Glasnevin produced in 1939 an extraordinarily rich crop of cones, permitting an intense pollination of the young ovules which were also plentiful in that year—and with spectacular results in 1940. The female tree bore hundreds of ripening fruits in dense clusters like rich bunches of grapes. The data obtained from the abundant 1940 collections were supplemented in 1941. The proximity of the Botanic Gardens allowed frequent collection over the critical periods, thus yielding a much closer series of stages than were possible in the study of, say, *Saxegothaea* (Looby and Doyle, 1939), in which case every collection necessitated a journey of about 80 miles.

OBSERVATIONS.

A. *Female gametophyte.* An immature ovule is shown in Fig. 1, no obvious indications of any archesporium being visible at this stage. During the third week of June most ovules are ready for pollination, and have assumed the typical podocarpean form. A young ovule at the average pollination stage

(Fig. 3) shows a small area of poorly differentiated archesporial tissue deep in the nucellus (Fig. 7). This archesporial tissue increases rapidly in size and distinctness, and, in the centre, one cell enlarges as a single gynospore mother cell surrounded by a tapetum (Fig. 8). Comparison between Fig. 3 and Fig. 6, which is a section, four days later, of an ovule at the mother cell stage, shows how rapidly the ovules are growing at this period. Shortly after pollination the micropyle develops typical closing cells for a short length along its distal end. Fig. 2 shows the type. The tapetum is a very conspicuous feature in *P. andinus*. It becomes increasingly obvious (Figs. 5, 6, 8, 9, 11) until the gametophyte begins to expand, when it gradually becomes confused with the disorganizing nucellar cells. It is therefore remarkable that Sinnott records its presence in all the podocarps in which young stages were secured *except* in the species of *Stachycarpus* examined—*P. spicatus* and *P. ferrugineus*. A photograph (his Pl. 8, 39) is given for the latter species at the stage when the functioning gynospore is enlarging. This actually seems to show a layer of cells, different from the nucellar cells, immediately surrounding the spore. As in any preparation the distinctness of the tapetum depends not only on its own degree of development, which shows some variation, but also on both the fixing and the staining used, it would be interesting to revise these two species. If only a very limited number of preparations are available for any stage it could possibly be overlooked.

Three gynospores are formed (Fig. 9), the lower of the two cells resulting from the first division dividing again. These stages are not illustrated, as no peculiar features could be seen in them. Of the three the lowest gives the gametophyte. Exceptions must be very rare, as in no case were more than three gynospores seen, and, in spite of the great number of ovules examined at all stages, in only one ovule were two gametophytes found. The number of spores formed in the conifer groups, three or four, shows little consistent relation, varying often from genus to genus. It is thus perhaps merely a matter of detail, but it is not clear how many typically occur in the two *Stachycarpus* species examined by Sinnott. He *writes* of them, that a "linear tetrad" is formed, but the only figure given, that already referred to for *P. ferrugineus, shows* the developing functioning spore and *two* abortive ones above it, thus suggesting three. The functioning spore becomes vacuolate and enlarges considerably while still uninucleate (Figs. 5, 11). This rather unusual phase, which has not been so far recorded, presents a striking similarity to the corresponding phase in *Saxegothaea* (Looby and Doyle, 1939, Fig. 2), although in *P. andinus* a projecting tip seems absent; nor was any structure later seen like the tiny tent-pole into which it developed in the cellular gametophyte in the former type. But Sinnott's figure, already referred to, of a germinating spore in *P. ferrugineus* almost certainly indicates the occurrence of such a phase in that species also. Development at this period is slow, this stage not being found till well into July, on occasion even not till the end of the month, the whole ovule steadily increasing in size (Fig. 5).

Free nuclear division soon begins, and the gametophyte develops to the stage shown in Fig. 4, in which condition it rests over the winter. Sinnott (1913) does not indicate in what stage his *Stachycarpus* species overwinter, but most probably they behave similarly. The gametophyte of *Saxegothaea* also overwinters as a small, more or less spherical, vacuolate prothallus with parietal nuclei (Looby and Doyle, 1939, Fig. 2G). In *P. andinus* this stage is reached about October, the actual resting stage being a little smaller than that shown in Fig. 4, which is from an ovule in April already wakening from the winter rest. This ovule further shows the very definite development of a wide zone of tissue, which surrounds the nucellus, and which continues above into the micropyle region. This tissue will later form the stone of the ripe seed, the outer tissue forming the flesh, although in both cases further expansion takes place before differentiation begins. The zone appears to be the integument proper, and can already be seen in quite young ovules. It shows with increasing distinctness in the stages illustrated in Figs. 3, 6, 5, 4, and 10. This series, including the still younger stage in Fig. 1, seems to support what is probably the simplest and most obvious interpretation of the fructification in *Podocarpus*. This interpretation considers it simply as an axillary structure bearing a single, attached, inverted, and partly embedded ovule, which is overgrown by the scale tissue, the so-called epimatium.

The exact dates of absolute winter rest were not determined. Increase in size is noticeable as March advances, and stages in typical alveolar formation (Fig. 12) are found by the latter part of April. Unfortunately during this period, and indeed until the gametophyte has become firmly cellular, the whole tissue is extremely delicate, much more so than is even usual at this notoriously difficult stage, and tends to collapse in any fixative. In *Saxegothaea* also the same difficulty was met with in an extreme form. As a result, although the development seems from the material available to be normal, it was difficult to secure stages good enough to photograph. Shortly after the alveoli have met in the centre, and the later typical cell formation has begun, two or three small archegonia can be seen in the upper part. One such is shown in Fig. 13. The actual earliest origin of these is not quite clear. When alveolar formation begins the small vacuolate gametophyte is circular, and obviously there is not room for all the alveoli to advance uniformly to the centre. Many close off early in the typical manner described in a recent paper on *Callitris* (Looby and Doyle, 1940), and are quite small and short, as is shown in the strip of young alveoli in Fig. 12. It is probable that it is two or three of these that actually become the archegonial initials. In most if not all conifers young alveoli, short or long, so function, and in many these can be distinguished from neighbouring vegetative alveoli by their slightly more definite appearance and the obvious lagging of wall formation in them. In the case of *P. andinus* the functioning ones are few and must be very small; they could not be identified till they actually began to show an archegonial appearance some little time after wall formation had begun. At the stage

shown in Fig. 13 outlines of the original alveoli could be still followed throughout the main body of the gametophyte. Sinnott's (1913) only statement on the archegonial origin is that each arises from a "single superficial cell."

At this time also begins a development essentially similar to that described for *Saxegothaea* (Looby and Doyle, 1939). Active periclinal divisions begin in the tissue surrounding the archegonia. An early stage is shown in Fig. 14, taken one section on the ribbon from a young archegonium. Extending in the long axis of the ovule the gametophyte rapidly becomes elliptical, the original centre remaining roughly as the lower focus of the ellipse. A more advanced stage is shown in Figs. 15, 16, the latter illustrating the upper region at a higher magnification. In this way is developed the characteristic cone which extends at maturity below the long archegonia, and which is shown rather coarsely in Fig. 24. The lower tip of the darker area still roughly corresponds to the original centre. The cells in the sub-archegonial area stain particularly heavily, but the cone extends up to the top, and expands well on both sides of the archegonia. The general form is thus essentially similar to that drawn diagrammatically for *Saxegothaea* in Fig. 3c of that paper, although in this form the cells flanking the archegonia tend to stain more heavily than in *P. andinus*. This arrangement of tissue, allowing for minor differences, corresponds to that described by Sinnott (1913) for *Dacrydium* and the species of the *Stachycarpus* group. It seems to be particularly distinct in *P. ferrugineus*. In these cases no indication is given of the nature of its development, but it is presumably like that described above. The cellular differentiation is in general similar to that described for *P. ferrugineus* by Sinnott and for *Saxegothaea* by Looby and Doyle.

As the gametophyte matures the two (or occasionally three) archegonia develop the form shown in Fig. 17. These again closely resemble those described for at least some species of *Dacrydium*, for *Saxegothaea*, and for the species of the *Stachycarpus* group. The jacket cells are not quite so distinct as in *Saxegothaea*. One point however needs further description. In Fig. 18, showing the upper part of the two archegonia in Fig. 17, it is clear that these are still young with the ventral canal nucleus not yet formed. The single-tiered neck can be seen in the left-hand one, and shows that they are already embedded at this stage. Flush, when young (Fig. 16), with the surface of the gametophyte tissue, the latter grows up around them, till at most only a very narrow canal extends from outside down to the necks. For all practical purposes this canal is obliterated by the meeting of the growing tissue, but its position may sometimes be seen clearly enough. This appears in Fig. 19 from a slightly older ovule. Both here and in Fig. 18 the thickening of the outer walls of the neck cells and the small dome-shaped cavity always left just above them are quite distinct. This embedding of the archegonia while still young is without exception the normal occurrence, and has taken place before the pollen tube has reached this area. The tube, at this time, has usually just bored through the nucellus below. The interest of this feature of structure will appear later.

It can also be seen from Fig. 18 that the neck cells, which are in one tier only, may be laterally placed in relation to the archegonial axis, and would be cut obliquely in an ordinary transverse section. This is a frequent feature, but fair transverse views of them are shown in Figs. 21, 22. The number varies from 10 to 15; 13 appear in Fig. 22, and 15 in Fig. 21. The former also shows the sequence of divisions and membrane formation. The original single cell divided into quadrants, each of which could divide into half-quadrants, in each of which again a final division could take place, giving a probable maximum of 16. In Fig. 22, in the lower right-hand quadrant the two half-quadrants have not divided; the next half-quadrant above also failed to divide—thus giving 13 neck cells in all. In Fig. 21, only one half-quadrant did not divide, giving 15. Actually the 16 maximum was never seen, but probably occurs. A further point of interest is shown in Figs. 18, 20. As can be seen from Fig. 18, the archegonial nucleus contains a large nucleolus. In sections stained for ordinary tissues this nucleolus is heavily overstained, so that its structure can only be made out in very lightly stained preparations. In Fig. 20 the large vesicular body is the nucleolus. The small loop to the left is really part of the nuclear membrane, which has contracted in from the outer edge of the nucleus proper, whose limits are shown by the clearer semi-lunar area on the left. Soon after the nucleolus has become really distinct the nucleus proceeds to the ventral canal division. Unfortunately the cytological fate and behaviour of this peculiar nucleolus could not be followed.

The mitosis leading to the formation of the ventral canal and egg nuclei, and which has not previously been recorded for any podocarp, is shown in Figs. 26 and 27. The first of these brings out its position and small size relative to the archegonium; the second, at a higher magnification, the coarsely granular appearance of the neighbouring cytoplasm. There also develops just at this time, in the centre of the upper part of the archegonium, a fairly large zone of more finely granular cytoplasm, into which the egg nucleus retires, leaving the ventral canal nucleus at the outer edge. Fig. 28 is from one of many stages showing this, although the finely granular zone does not come out well in the reproduction. The same appears at a slightly later stage in Fig. 29. Since the mitosis is usually oblique and a little away from the neck, the ventral canal nucleus usually lies to one side of (Figs. 28, 29 and 49), and only occasionally opposite to (Fig. 48), the neck cells. As in so many other conifers, it may be very evanescent, may persist almost up to fertilization, or, very rarely, may enlarge and lie free in the cytoplasm. The egg nucleus enlarges rapidly (Figs. 42, 43, 48, 49), the finely granular cytoplasm forming a neat sheath around it. Two mature archegonia, ready for fertilization, are shown in Fig. 30. In the one on the right the egg nucleus is lying symmetrically, and is cut practically medianly; the large size of the nucleus and the surrounding sheath are very characteristic at this stage. Many scattered vacuoles, large and small, still persist up to fertilization, and, in addition to the fine and coarse granules, larger deeply-staining but irregular-shaped masses, probably protein in nature, occur frequently. These are

however hardly regular enough to be classed as "protein vacuoles." These archegonia resemble closely those described by Sinnott (1913) for *P. ferrugineus* and *P. spicatus,* except that, in these, vacuoles seem a little less numerous. The archegonia of *Saxegothaea,* while similar in general, lack the numerous small vacuoles and apparently also the sheath round the egg-nucleus, the "protein vacuoles" being, on the other hand, more definite.

The gynospore membrane is thick and definite. In Fig. 23, an ovule in rough T.S., the membrane appears as a thick dark line between nucellus and gametophyte, the latter with the characteristic two archegonia. The membrane itself is illustrated in Fig. 25, and shows clearly the inner continuous layer and the outer coarsely fibrillate layer. *Saxegothaea* (Looby and Doyle, 1939, Fig. 3c) possesses a membrane with similar structure. It is possible that the inner continuous membrane may be really double. At the gametophyte apex the membrane thins out rapidly over the archegonial area (Fig. 29).

Considerable changes take place in the meantime in the vegetative tissues of ovule and epimatium. A comparative study in *Podocarpus* of the changes taking place in these tissues up to full maturity has not yet been made, although it would probably yield fruitful results, but, in spite of their interest, a detailed account of *P. andinus* is here omitted except for brief reference to Fig. 10. To facilitate cutting and penetration of fixative this ovule has been sliced down front and rear, but the micropilar region is still intact. The tissues round the epimatial canal have grown and closed it. Within is the micropyle proper. Active division has considerably increased the width of the micropylar part of the integument, but on the outer sides towards its distal part a number of layers of large cells, rather palisade-like in shape, and probably containing resin, have developed. This tissue stops proximally just where the integument fuses with the scale tissue, and distally thins out at the small dark area at the actual micropyle. This dark area is the original group of closing cells shown in Fig. 2. The small-celled part of the integument, sharply marked off from the scale and epimatium tissue, swings completely round and over the nucellus and gametophyte. At the base, to the left in Fig. 10, there can be made out in order from the outside, looser scale tissue, the compact integument tissue, and the nucellar tissue. Actually the scale and integument tissue are separated by a very narrow zone of cells which differ from both. It does not appear in this figure. The loose tissue on the sides of the micropylar part of the integument probably corresponds to the resin tissue described by Gibbs (1912) for *P. ferrugineus* (another of the *Stachycarpus* species), although in this case it ranges right down to the base of the ovule. The narrow zone referred to above probably represents this in *P. andinus.*

B. *Male gametophyte.* This account starts with the shed grain when first found in the micropyle shortly after pollination (Fig. 31). The prothallial cells (or nuclei) are grouped at one side in a small dense clump, which, when lightly stained, can be resolved with high magnification into a number of

separate nuclei, the exact number being difficult to determine at this stage. The nuclear clump in the centre of the upper grain, for example, can be shown to be composed of three nuclei—presumably the tube, body, and stalk, nuclei. The grain rests for about three weeks, sometimes much longer, before germination, which is direct, the outer wall of the grain being retained as in the Pinaceae. As the tube projects the prothallial nuclei enlarge and gradually become free in the cytoplasm, all the nuclei except the body nucleus then passing down the tube, which only penetrates a short distance into the nucellus. It usually branches at the tip. Fig. 33, taken about six weeks after germination, shows a tube and grain cut to one side of the body cell. Eight nuclei are shown, two more appearing on changing the focus—there are thus at least eight prothallial nuclei. Fig. 34 is the same grain from a near-by section on the ribbon to show the body nucleus surrounded by zoned cytoplasm. Growth in this tube is nearly complete, but ordinarily a slight further penetration and a widening of the branches will take place. Fig. 41 is included merely to show the approximate maximum penetration and branching of the tube. The summer growth is probably complete about mid-August. The gametophyte then enters into a state of apparent dormancy, from which it will not waken till the middle of the succeeding May.

This behaviour of the tube and nuclei is again paralleled in *Saxegothaea*—a short summer growth, a long winter rest, the tube tip penetrated only a short distance into the nucellus, the sterile nuclei strung along the tube, and the body nucleus overwintering in the grain. In *Saxegothaea* the prothallial nuclei are, of course, fewer, and lie free in the cytoplasm of the grain before shedding. Sinnott (1913) does not state in what stage the male gametophytes in *P. spicatus* and *P. ferrugineus* overwinter. He merely says that the sterile nuclei precede the body cell down the tube, but from his drawing, Pl. 5, 4, it is practically certain that these species resemble *P. andinus*. *Dacrydium cupressinum*, in which development is spread over two seasons, also shows a similar condition of tube and nuclei during the winter rest (Stiles 1912). It is perhaps of interest that in this paper by Stiles is to be found the only reference in the literature to any gametophytic phase in *P. andinus*. He mentions that up to eight prothallial cells may be found in the grain, which agrees with the maximum number of nuclei seen in the later stages here described. He states further however that, like all other podocarps, the prothallial nuclei in this species come to lie free in the cytoplasm of the grain at the shedding period. Clearly in Fig. 31 it cannot be said that the prothallial nuclei lie obviously free in the cytoplasm in the same sense as they lie free in *Saxegothaea*, for example. This apparent discrepancy raises questions concerning the intimate details of development in the grain while still in the pollen sac, but as these questions do not bear directly on the matter of this paper their discussion can be postponed till certain further data are available. As in other aspects of podocarpean life-history many points still need critical revision in these phases also.

The tube figured in Fig. 32 is from an ovule gathered in the succeeding April, practically nine months later than that in Fig. 33. Although bent, as most of the tubes naturally are, and so only partly in focus, it shows the general stage about ten months after pollination. During the latter part of April and the first part of May the body nucleus enlarges slowly, its associated protoplasm also becoming more compact, but the first signs of awakening activity were not secured till May 29, when the first preparation was got in which the body cell had obviously begun to move from its winter position in the grain (Fig. 35). Development at first is not rapid, so that in the first week of June many stages were secured like that in Fig. 36, which shows a cluster of tubes, in two of which the body cell has moved well down. It also seems clear, in spite of the branching of the tube at the tip, that all, certainly most, of the sterile nuclei proceed down one branch, usually the most advanced branch, and that the body cell follows in the same path; in later stages a number of sterile nuclei are always found in front of it. The cytoplasm of the body cell is still rather ill-defined from that of the rest of the pollen tube.

Subsequent development is more rapid, and fertilization is commonly effected within ten days of the body cell reaching below the level of the nucellar surface. Special attention was given to this subsequent history of the tube and the body cell. In the paper on *Saxegothaea* (Looby and Doyle, 1939) a short survey was given (pp. 110–111) of our knowledge of the male cells in the podocarps as a whole, following the demonstration of the occurrence in that plant of two equal male cells. With the exception of *Microcachrys*, which in any case needs revision, the podocarps have been taken to be characterized by an extreme inequality of the male cells, the smaller of which, in *Podocarpus* proper, is said to be represented merely by a nucleus, partly or wholly extruded from the cytoplasm of the functioning cell. The survey showed that this concept, while probably quite correct, is actually based on such meagre evidence that a critic might readily reject it. In most cases only fragmentary data are given; in no case is a complete history of the tube from germination to fertilization available, and the only really cogent evidence, that supplied by Coker in 1902, was derived from the examination of only two pollen tubes. To secure stages in the male gametophyte of *P. andinus* was thus advisable, especially as the species is one of the simplest in the genus.

As the tube begins to grow more rapidly it penetrates the lower half of the nucellus, in which the cells are compact and rather densely filled with starch grains. Here the tube is narrow, as if certain difficulties in penetration existed, and the whole body cell becomes drawn out. Staining at this stage is also difficult, the dense cytoplasm colouring so intensely as to obscure the nucleus. A typical view, at a low magnification, is given in Fig. 37, merely to show the condition. In this the small upper darker area of the dense mass in the tube is the body nucleus, which is actually in the prophase of its division, although it proved impossible to record this photographically at any magnification. A number of nuclei, also not shown, are embedded in the

slightly lighter protoplasm in front. As the tip gets through the nucellus the whole tube seems freer to widen and expand a little, the body cell becoming more elliptical and normal in shape. At this stage the body nucleus divides. The difficulty of contrast staining still continues, however, so that the only mitosis which could be clearly recorded (Fig. 38) was in a tube with a marked bend just at this level. This mitosis in the body cell, recorded here for the first time in any podocarp, occurs at the upper part of the body cell. The lower of the two nuclei formed moves slowly to the centre of the cytoplasm, and becomes gradually larger than the other, which remains in position above. By this time the contents of the tube have reached at least the level of the lower surface of the nucellus. Figs. 39 and 40 show two successive sections of the same tube. It is thus already clear at this stage that an extreme inequality of the male cells will result. Fig. 39 shows the principal male nucleus lying in the main body of the cytoplasm; the second male nucleus, which will not function, is apparently almost extruded already from the main cytoplasm (Fig. 40), although it is doubtful if extrusion ever really occurs.

Before, however, commenting on the various stages reproduced to illustrate the maturing of the male cells a word is necessary on the growth of the pollen tube. Sinnott (1913) definitely states for *P. spicatus* and *P. ferrugineus* that, while still young, the pollen tubes lie over the archegonia, which are still almost flush with the surface of the prothallus. The surrounding gametophyte tissue then grows up round the pollen tubes, enclosing them in a deep cavity, at the bottom of which the neck cells lie. The result is figured for *P. ferrugineus* in his Pl. 5, 2. He further states that any archegonium not reached in time by a pollen tube will also become embedded, and cannot then subsequently be fertilized. In view of what can readily be followed in *P. andinus*, Sinnott's account is almost certainly the result of incorrect interpretation from too few preparations. In *P. andinus* the tube burrows between gametophyte and nucellus till it comes to lie over the thin area of the gynospore membrane (Fig. 29) above the position of the archegonia already deeply embedded. It bores through the membrane and burrows down approximately through the canal left above the necks by the upgrowth of the gametophyte tissue. In Fig. 42, which includes the full length of the archegonia, the tube, indicated really by its contents, can be seen pushing into the canal above. Fig. 43 shows the same tube and the upper part of the archegonia at a higher magnification. Fig. 44, from the next section on the same ribbon, shows clearly the boring tip of the tube. Stages in the further penetration to the necks appear in Figs. 45, 46, 47. On reaching the neck the end of the tube spreads out over the top of the archegonium, to produce the effect shown in Fig. 48. Although the tube contents in this preparation are badly contracted it is included here, as it shows this splay effect so well, but the same appearance is also clearly seen in Figs. 49, 50, 51, 52, 55 and 56. The cells over the top of the archegonium are compressed and presumably digested away till the neck cells finally remain swung from the sides by a membrane only. From Sinnott's

rather diagrammatic drawing, which corresponds in *P. ferrugineus* to this group of figures, we are convinced that the behaviour in this species, and presumably also in *P. spicatus,* would prove to be essentially similar to that in *P. andinus,* if a suitable series of stages were available.

Further, this behaviour in *P. andinus* definitely helps to clarify the corresponding stage in *Saxegothaea* and *Phyllocladus.* In the former, when ready for fertilization, the archegonial necks are sunk, but only in an open shallow pit. The figure, Pl. 2, 10, in the paper by Looby and Doyle (1939) shows a fairly common condition in *Saxegothaea,* the presence of only one effective pollen tube, so that an undisturbed archegonium can be found beside one about to be fertilized. Obviously the pollen tube has grown down, clearing away the lateral cells and pressing the resistant archegonial (?) membrane in front of it with the neck cells suspended in the centre. This arrangement of the neck cells at this stage must have been characteristic of the primitive podocarpean type. It is shown in species of *Dacrydium* (Stiles, 1911) and *Phyllocladus,* in which latter genus it was first recorded by Young (1910). Young was of the opinion that the condition was produced not only by pollen tube pressure, but also by upgrowth of prothallial tissue round the tube. Every figure of these stages published in Young's paper shows, however, an archegonium reached by a pollen tube, no comparative control in the form of an archegonium without a tube, such as is available in *Saxegothaea,* being discussed. As Young's material, though apparently very fertile, was not plentiful, and as the archegonia, when first reached by a pollen tube, are also sunk in a shallow open pit, it seems reasonable to infer that the behaviour in these two types is essentially similar and differs from that in the *Stachycarpus* species only in the shallower position of the archegonia.

When the tube begins to bore into the gametophyte tissue the male cell complex (Fig. 43) is usually only a little further advanced than the stage shown in Figs. 39, 40. The functioning nucleus is clearly seen in the centre, the smaller, not quite in focus, above. In every case in which fixation and staining were satisfactory, this small nucleus could be seen to be still embedded in the cytoplasm. This point will be returned to shortly. A clump of sterile nuclei, which always lies in front of the male cells, can be seen in Fig. 44. In the next figure, Fig. 45, the smaller male nucleus is barely indicated above, but there is seen below a zone of looser cytoplasm, which occupies the anterior part of the tube, and from which the male cell cytoplasm seems fairly well delimited. Actually, however, the small dark specks, the sterile nuclei, below the visible male nucleus are themselves embedded in cytoplasm, from which only later will the male cell proper be separated. In Fig. 46 the male cells are older, the nucleus of the larger one now showing the characteristic nucleolus—the dense dark area. The pollen tube in Fig. 47, though a poor one, is included, as it shows the smaller nucleus in front of the other. This is fairly common (10 per cent. of cases?), but its origin in relation to the body cell division was not determined. Maturing male cells are shown in Figs. 49, 50. Here the

anterior cytoplasm, shown in Fig. 45, has spread out over the archegonial necks; the zone of cytoplasm containing the sterile nuclei appears a little distinct from the male cells; and the cytoplasm of the functioning male cell has become more clearly delimited. The male cells are still further advanced in Fig. 51. The zone in which the sterile nuclei are embedded is definitely distinct, and in fixation tends to separate from the male cells. The delimitation above between the smaller and larger male cells is still a little indefinite.

P. andinus seems definitely to form two male *cells*, although these are very unequal in size. The real nature of the smaller male cell sometimes can be seen very clearly, as in the stages illustrated in Figs. 57, 58, 59, and 52. The first of these figures shows a definite delimitation of an area of cytoplasm surrounding the smaller nucleus above. The other nucleus was only partly included in this section, and does not show in this plane of focus. It is unusual to notice so clear a separation in so young a stage, or to find so relatively large an amount of cytoplasm associated with this smaller nucleus. Fig. 58 is more typical of the advanced stages. The functioning male cell is almost mature, as shown by the extraordinary large and dense nucleolus and the indications of an outer membrane. The narrow belt of cytoplasm shows distinctly round the smaller nucleus, which also contains a similar nucleolus. In this case the area containing the sterile nuclei is overlying the lower part of the larger cell, and obscures its lower boundary. In Fig. 52 this zone of protoplasm has contracted away, leaving isolated the functioning male cell, which is just mature. In this case the second male cell lay in the same position as that shown in Fig. 47. Its nucleus is in a neighbouring section, but here there appears a thin projecting vesicle at the lower right corner, which is the thin layer of cytoplasm which surrounded it as in Fig. 58. The smaller complete male cell is also clear in Fig. 59, from a stage immediately before fertilization. The large male cell is again thrown out of focus in order to emphasise the line of demarcation between the two. Admittedly in cases where fixation was poor the presence of a protoplasmic layer round the second male nucleus was not so certain, but it is considered probable that in all cases two very unequal male *cells* are present.

A striking feature in the male cells, particularly at maturity, is the enormous nucleolus. As the plate figures show, it colours intensely, but a complex vesicular structure (Fig. 60) can be seen when very lightly stained. In this case it is cut tangentially, as the vesicular appearance is then a little more definite for photographic record. The nucleolus of the smaller male cell is similar.

Development proceeds in a very uniform manner in most cases, irregularities or departures from the described sequence being few. Thus in one case the male cells developed large nucleoli while still in the nucellus (Figs. 53, 54). This was from one of the successful pollinations with Rostrevor pollen, which may perhaps mature more rapidly. On the other hand, the male cell in Fig. 29, from Glasnevin pollen, is also somewhat advanced for a tube in

this position. The smaller male cell in this case was not in the plane of this section. Its absence rather over-emphasizes the distinctness of this functioning one. There occurred, however, one feature which could not be interpreted with certainty. The tube in Fig. 55 seems to contain two subequal male cells in close contact, the nucleus of the upper being a little indistinct. The preceding section of the ribbon, not here reproduced, shows a small area of the lower part and a fairly definite circular tangential section of the upper. The section succeeding Fig. 55 on the ribbon is shown in Fig. 56. The main body here is a section of the larger lower area in Fig. 55 cut tangentially to the nucleus, but at the lower right corner lies a body which shows every appearance of being a typical smaller male nucleus in a forward position. At least three obvious interpretations could be put forward, including the possibility of this being a reversionary development of two equal male cells, but any suggestion based merely on this single equivocal occurrence is really valueless.

Fertilization is now due to take place, and will be described, with the later stages, in a succeeding paper.

Summary.

The female gametophyte, and the male gametophyte from the lodgment of the pollen grains in the micropyle, are described, for *Podocarpus andinus*, up to the fertilization stage. In form, the mature female gametophyte closely resembles that already described for *Saxegothaea* and certain species of the *Stachycarpus* group. Earlier stages are also similar to those in *Saxegothaea*. Both show a single mother cell giving three gynospores, a distinct tapetum, marked increase in size and vacuolization of the functioning spore before the first nuclear division, overwintering as a small circular vacuolate prothallus, and development around the young archegonia of a meristematic cone, which forms most of the mature gametophyte. *P. andinus* only differs in the deep embedding of the archegonia while still young.

The pollen tube penetrates only a short distance into the nucellus, and overwinters with up to ten nuclei strung along the tube—the stalk and body nuclei and up to eight prothallial nuclei—leaving the body nucleus with associated cytoplasm resting in the grain, from which position it does not move till about eight months later. Except for the smaller number of sterile nuclei *Saxegothaea* shows a similar resting phase.

Special attention was given to the later development of the pollen tube and the body cell to clarify the nature of the male gametes in one, at least, of the simpler species of *Podocarpus*. The tube bores down through the overlying tissue to reach a deep-seated archegonium, over the top of which it splays out. The origin of the isolated position of the neck cells, recorded for many podocarps, is explained. Division in the body cell is recorded for the first time in any podocarp. A series of stages in the organization of the male gametes is described. There develop two unequal male *cells*; the smaller may be very

definitely delimited from the larger functioning one. Only in this feature of the inequality of the male cells do the gametophytes of *P. andinus,* in general, differ from those of *Saxegothaea.*

EXPLANATION OF PLATES.

PLATE 6.

1. L.S. very young ovule and subtending bract. June 6. × 45.
2. L.S. micropyle to show position and nature of closing cells. June 29. × 150.
3. L.S. ovule at pollination to show general form and size. June 19. × 45.
4. L.S. ovule, partly cut away, to show circular vacuolate gametophyte. April 19. × 20.
5. L.S. ovule at stage of vacuolate uninucleate gametophyte with good tapetum. July 30. × 45.
6. L.S. ovule at stage of gynospore mother cell; tapetum distinct. June 23. × 45.
7. Part of 3 at higher magnification to show young archesporial tissue deep in nucellus. June 19. × 150.
8. Gynospore mother cell with surrounding tapetum and nucellar cells. June 23. × 300.
9. Row of three gynospores with surrounding tapetum. June 29. × 300.
10. L.S. older ovule sliced away on two faces to show developments in integument; also cellular gametophyte with young archegonia. May 28. × 20.

PLATE 7.

11. Uninucleate vacuolate gemetophyte, somewhat contracted, with surrounding tissue. July 30. × 140.
12. Early alveolar formation. April 23. × 300.
13. Very young archegonium with surrounding tissue, recently derived from alveoli. May 12. × 140.
14. Initiation of active periclinal divisions in tissue surrounding the young archegonia; traces of recent alveolar formation still visible. May 12. × 140.
15. Young gametophyte, with developing archegonia; explanation in text. May 28. × 45.
16. Archegonial area of 15 at higher magnification. May 28. × 65.
17. Complete maturing archegonia just before formation of ventral canal nucleus. June 10. × 45.
18. Upper portions of same showing nucleus, necks and overgrowth of gametophyte tissue. June 10. × 140.
19. Area just above the neck of an older archegonium to show the narrow canal formed by the overgrowth of the gametophyte tissue. June 19. × 300.
20. The peculiar nucleolus of the archegonial nucleus at stage of Fig. 18. June 10. × 800.
21. To show 15 neck cells. June 10. × 140.
22. To show method of division of primary neck cells. June 10. × 600.
23. T.S. of part of gametophyte showing two archegonia with jacket-cells; the surrounding nucellar tissue and the thick gynospore membrane. June 10. × 45.

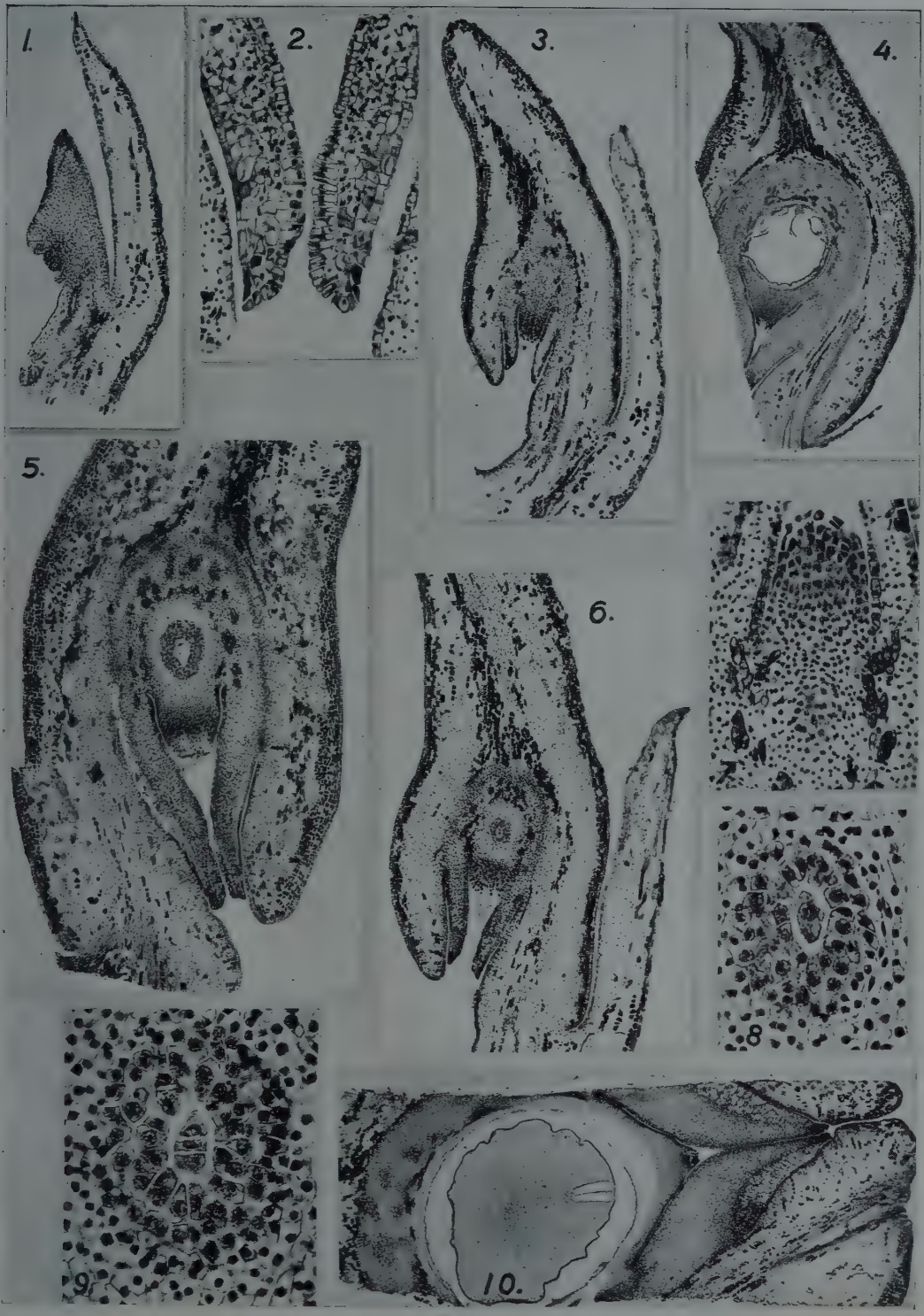
24. L.S. complete gametophyte to show the cone of tissue stretching below the archegonia to position of the orginal centre. June 10. × 20.
25. Gynospore membrane to show inner continuous layer and outer fibrillate layer. June 10. × 1600.

PLATE 8.

26. Division cutting off ventral canal nucleus to show position and size relative to archegonium. June 14. × 140.
27. Same at higher magnification showing the coarsely granulate cytoplasm. June 14. × 300.
28. Egg nucleus, shortly after the division, retiring into the central more finely granular zone of cytoplasm; ventral canal nucleus still visible. June 14. × 300.
29. Upper portion of archegonium at slightly later stage to show egg nucleus, now within the finely granular zone; ventral canal nucleus; the pollen tube, containing a rather advanced male cell, just before its penetration of the thin area of gynospore membrane above position of deeply sunken archegonia. June 13. × 140.
30. Two mature archegonia; on right egg nucleus, with finely granular sheath, is cut medianly; pollen tubes down to necks in each case; tube on left cut tangentially but includes a mature male cell. June 17. × 140.
31. Pollen grains in micropyle. June 27. × 300.
32. Resting winter stage of pollen tube, the sterile nuclei strung along the tube, the body-cell left behind in the grain. April 23. × 300.
33. Pollen grain and tube shortly after germination, 8 sterile nuclei passing down, body cell in next section (33). July 30. × 600.
34. Young body cell in same grain, as 33. July 30. × 600.
35. Body cell just moving from resting position in the grain. May 29. × 300.
36. Cluster of pollen tubes in two of which the body cell has moved well down. June 6. × 140.
37. Pollen tube just through the nucellus, detailed description in text. June 13. × 150.
38. Division of body cell nucleus. June 13. × 600.
39 and 40. The two male nuclei shortly after the division of the body nucleus. In 39 the functioning male nucleus and in 40 the non-functioning one; body cell cytoplasm poorly differentiated from the rest of the protoplasm of the tube, especially anteriorly. June 13. × 300.
41. To show branching of pollen tube; with 32, shows maximum development of tube at resting stage. April 23. × 140.

PLATE 9.

42. Full length archegonia almost mature; pollen tube with young male cells just penetrating gametophytic canal above. June 13. × 45.
43. Tube boring down to neck; functional male cell still quite young; second male nucleus just visible above the larger functional one. June 13. × 150.
44. The boring tip of the pollen tube; sterile nuclei visible; from same tube as 43 but a section further on. June 13. × 300.
45. Another tube just down to level of archegonial necks; male cell still young; upper non-functional male nucleus barely indicated above; male cytoplasm not yet fully marked out from rest; sterile nuclei bunched in what appears to be the lower part of the male cell cytoplasm. June 13. × 300.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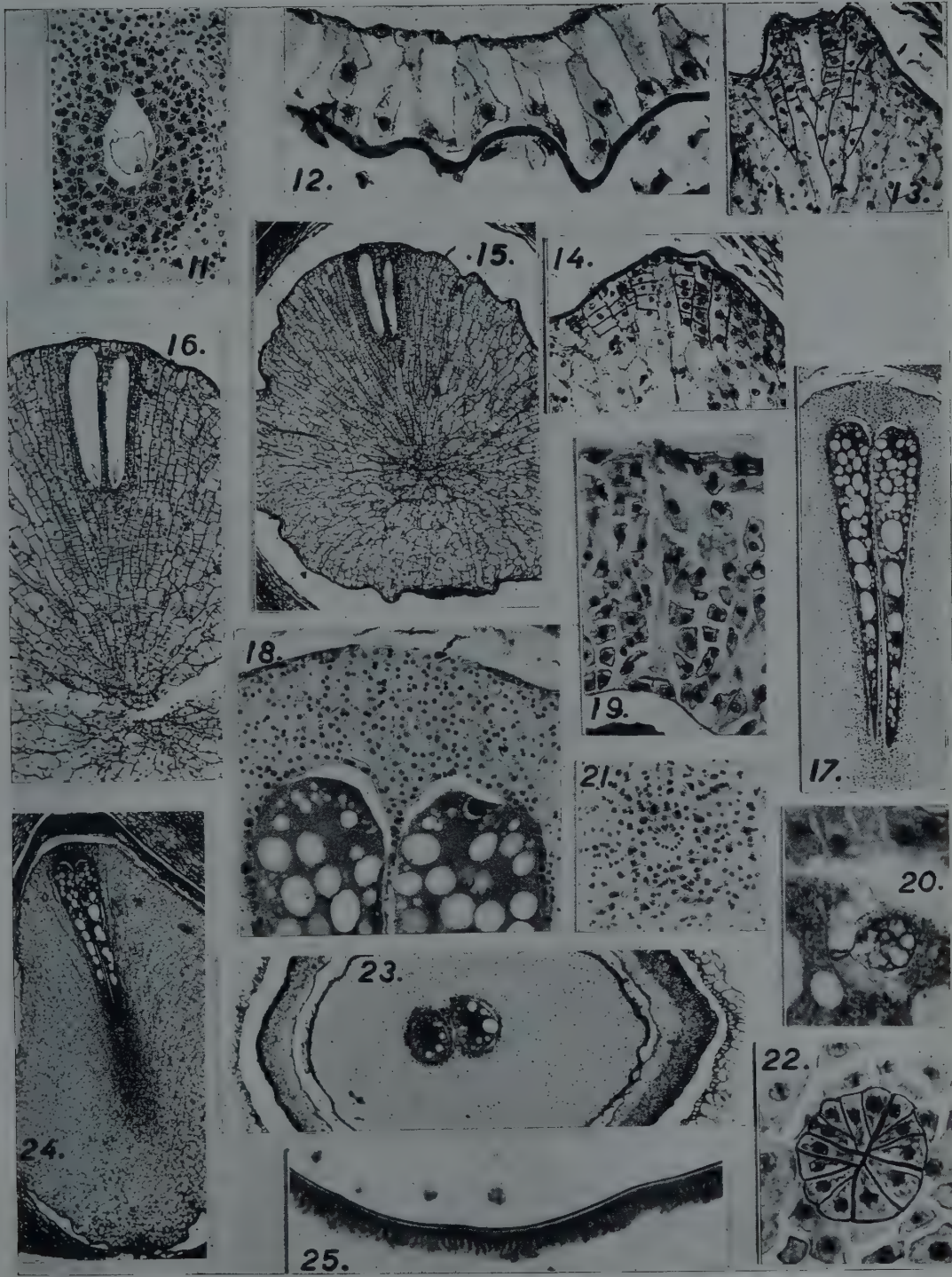
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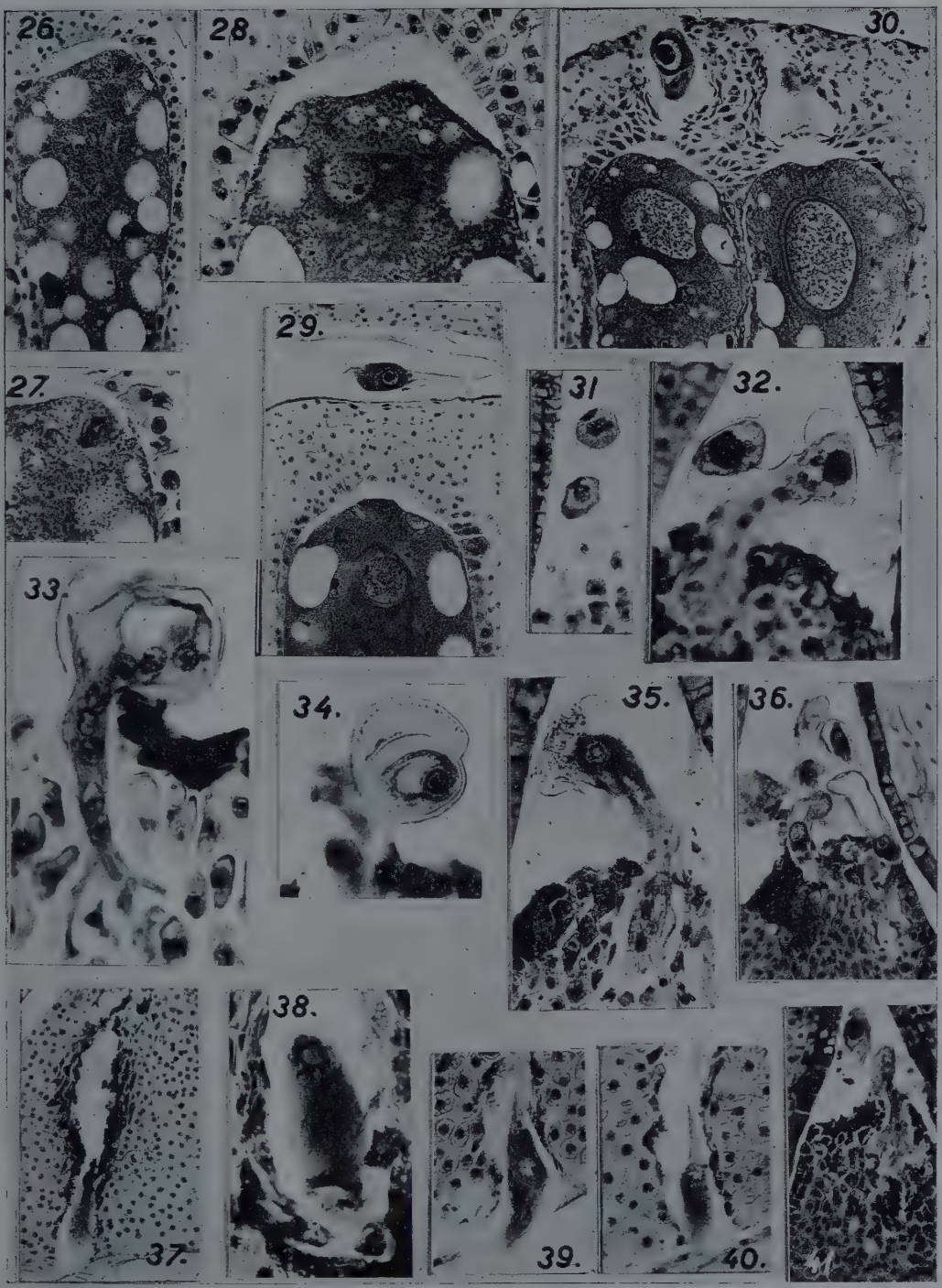
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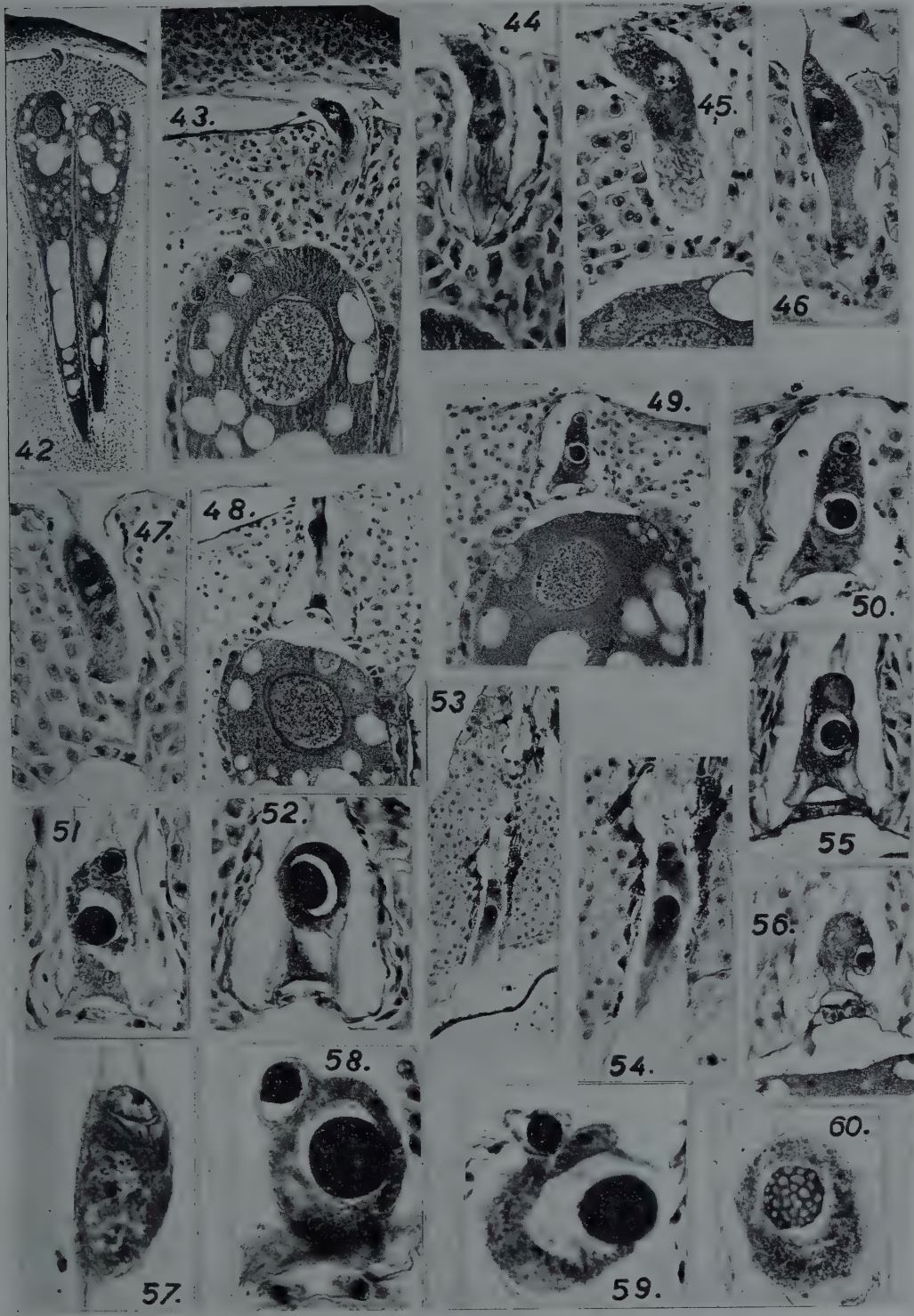
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46. Slightly older male cell; nucleolus of functioning nucleus enlarging; sterile nuclei, probably not visible in reproduction, bunched as in 45; non-functioning male above. June 13. × 300.
47. Similar stage to show non-functioning male nucleus embedded in cytoplasm in front of the functioning one. June 13. × 300.
48. To show how the end of the pollen tube splays out over the top of the archegonium to leave neck cells attached by a membrane to the side; tube contents poor, but egg nucleus, surrounded by finely granular sheath, and ventral canal nucleus clear. June 16. × 150.
49. Maturing male cells over archegonium; ventral canal nucleus still visible. June 13. × 150.
50. Male cells at a higher magnification, cytoplasm of functioning one becoming more distinctly limited from zone below containing the sterile nuclei. June 13. × 300.
51. Male cells almost mature showing the enormous nucleoli; the smaller nucleus sheathed in protoplasm; the functioning cell clearly distinct below from the zone containing the sterile nuclei, several of which are visible. June 13. × 300.
52. Male cell mature. In this case the smaller male cell (which appears in another section) was below; here its thin protoplasmic sheath is shown at the lower right corner of the male cell. June 17. × 300.
53. Male cells with large nucleoli formed while still in nucellus. June 14. × 150.
54. Same at higher magnification. June 14. × 300.

55 and 56. Unusual male cell complex. See text for explanation. June 15. × 300.

57. Young male cells, nucleus of larger only faintly visible in this section, which shows the definite delimitation of the smaller as a distinct male cell. June 13. × 600.
58. Mature male cells, the smaller with its distinct cytoplasmic sheath; the delimitation of the larger and the membrane surrounding it thrown out of focus a little to secure the smaller. June 13. × 600.
59. Similar stage from a male cell complex immediately before fertilization, functioning male cell considerably out of focus in this case. June 16. × 600.
60. Nucleolus of a functioning male cell cut tangentially to show its structure. June 13. × 600.

REFERENCES.

BUCHHOLZ, J. T. (1936).—Bot. Gaz., **98**; 135–146.
——————— (1941).—Bot. Gaz., **103**; 1–37.
COKER, W. C. (1902).—Bot. Gaz., **33**; 89–107.
GIBBS, L. (1912).—Ann. Bot., **26**; 515–572.
LOOBY, W. J., and J. DOYLE (1939).—Sci. Proc. Roy. Dub. Soc., **22** (9); 95–117.
——————— (1940).—Sci. Proc. Roy. Dub. Soc., **22** (24); 241–255.
SINNOTT, E. W. (1913).—Ann. Bot., **27**; 39–82.
STILES, W. (1911).—New. Phyt., **10**; 342–347.
——————— (1912).—Ann. Bot., **26**; 443–514.
TAHARA, M. (1941).—Sci. Rpts. Tokohu Imp. Univ. Ser. IV, **14**; 91–98.
YOUNG, M. (1910))—Bot. Gaz., **50**; 81–100.

No. 23.

REPORT OF THE RADIUM COMMITTEE FOR THE YEAR 1943.

[Read MAY 23. Published separately JUNE 8, 1944.]

647 tubes containing 5077 millicuries were issued in 1943, as compared with 1109 tubes containing 6648 millicuries in 1942.

Mr. W. M. Hynes was elected to the Radium Exhibition for 1943–44.

Reports from users record the treatment of the following cases with radon or radium element during 1943:—

	Malignant.	Non-malignant.	Total.
Dr. O. Murphy, St. Vincent's Hospital, Dublin ...	80	82	162
Dr. O. Chance, Richmond, Sir Patrick Dun's, Rotunda, and Coombe Hospitals, Dublin ...	24	5	29
Dr. Bethel Solomons	—	3	3
St. Anne's Hospital, Dublin	136	—	136
Totals,	240	90	330
Totals for the same users in 1942	234	108	342

The reports show that for cases of malignant disease first seen during each of the years 1941, 1942, and 1943, the following methods of treatment were adopted:—

	1941.	1942.	1943.
Cases seen	1204	928	964
Cases treated, Total	1015	730	779
" " by Surgery alone	176	54	43
" " by Radium alone	222	184	187
" " by X-Rays alone	457	406	464
" " by Surgery and Radium	30	17	13
" " by Surgery and X-Rays	68	26	32
" " by Radium and X-Rays	60	41	35
" " by Surgery, Radium, and X-Rays ...	2	2	5

As there is no appreciable decline in the number of cases receiving radium treatment in 1943, as compared with 1942, it appears that the decreased use of radon must be attributed to an increase in the use of radium element.

Dr. Murphy reports the radiological treatment of the following cases:—

Malignant.—Carcinoma of lip, 6; ear, 2; floor of mouth and tongue, 5; breast (secondaries), 4; bladder, 1; uterine body, 2; uterine cervix, 12; skin surface, 14. Rodent ulcers, 36. Total 82, of which 80 were treated with radon or radium element.

Non-malignant.—T.B. Peritonitis, 8; Uterine hæmorrhage, 4; Angiomata, 18; Lupus vulgaris, 2; Lupus erythmatosis, 2; Keloids, 3; Warts, 46. Total 82; all treated with radon or radium element.

Dr. Chance reports the radiological treatment of the following cases:—

Malignant.—Carcinoma of skin, 21; lip, 20; tongue 11; cheek, 2; palate, 2; tonsil, 2; alveolus, 3; larynx (extrinsic), 2; pharynx, 1; glands of neck, 4; breast (*a*) operable, 16, (*b*) inoperable, 3, (*c*) post-operative recurrence, 3; cervix, 3; fallopian tube, 1; vulva, 1; bladder, 1; lung, 1; rectum, 2; pituitary gland, 1; spinal cord, 1; post-cricoid carcinoma, 3. Sarcoma (various), 5. Rodent ulcers, 43. Total 132, of which 24 were treated with radon or radium element.

Non-malignant.—Hodgkin's disease, 1; Myelogenous leukæmia, 1; Nævi, 3; Metropathia hæmorrhagica, 3; Tubercular peritonitis, 2; Tubercular glands, 1; Keloids, 2; Exuberant scar tissue, 4; Infected plantar warts, 8; Osteo-arthritis, 3; Various, 4. Total 32. Of these the only cases treated with radon were the two T.B. peritonitis, one nævus, and two various.

No. 24.

STUDIES IN PEAT.

Part 11.

PEAT-TAR OILS.

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[Read May 23. Published separately June 15, 1944.]

While the thermal decomposition of peat and bituminous coal would appear to be closely related operations, yet from every consideration practically all advances have been made with the latter material. To take one particular example—the coal-tar distillates from low temperature carbonisation show an extensive literature, while the corresponding peat-tar oils have been examined to a very limited extent.

Some claims have been made for the disinfectant properties of peat-tar fractions by various authors, though experimental evidence to justify these claims is seldom recorded.

Owing to the recent use of peat in gas plants and in charcoal manufacture, with the possible availability of peat-oils, this would appear to be an opportune time for a more extended study of these oils. Except for a preliminary note by Morgan and Scharf (1), very little experimental work appears to have been carried out on such products, especially from Irish peats. In these early reports, no indication is given of the nature of the peat, and there is some evidence that wood and other combustibles were also used in the producer-plant. Part of the lighter oils had also been drained away, and in some cases the samples had undergone changes by long exposure to the atmosphere before examination.

Distillation of Peat.

The peat was air-dried, machine-won peat from Lyrecrumpane, County Kerry. A considerable amount of work has already been carried out at University College, Cork, on this peat in connection with the work on peat waxes (2). Before being placed in the retort the peat was

crushed to pass a sieve of twenty meshes to the inch. The distillation was carried out in an iron retort, gas-heated, and was effected at the comparatively low temperature of 300°–350° C. The condensation system employed was that devised by Donnelly and Reilly (3) to eliminate the disturbing "tar-fog" effect. These authors found that a good fog-trap was provided by a column of tightly packed glass-wool.

Two distillations were carried out, a charge of 1,000 g. of peat being used in each case. When the temperature reached 100° C. it remained constant for about 40 min., water coming over meanwhile also very small quantities of volatile oils which had been steam-distilled. The heating was increased, and the temperature rose rapidly to 300° C. and then slowly to 350° C. The yield of tar-oil distillate from a number of experiments varied from 7·6 to 8·2 per cent. calculated on air-dried peat. Davis (4) mentions that the average yield of tar from peat distilled in retorts is 4·5 per cent. The low temperature carbonisation greatly increases the yield of tar by preventing the cracking of the heavier tar and oils to gaseous matter.

The distillates from the separate experiments were mixed and re-distilled. A further small separation of water was thereby effected. The distillates were extracted with sodium hydroxide solution, and the phenols thus dissolved were re-precipitated with dilute sulphuric acid and then re-distilled to eliminate resinous impurities. For the purposes of examination two fractions were collected, 120°–210° C. and 210°–300° C. The volatile oils of the first fraction were light brown almost yellow in colour, those of the second being of a darker shade. On standing both fractions reverted to an opaque blackish-brown.

The weight of alkali-soluble substances prepared by the above method was 2·14 per cent. of the original air-dried peat. The tar oil was not further refined, since in this form it rapidly gives emulsions. A vacuum distillation of the alkali-soluble fractions was, however, carried out, with results which will be referred to later.

Procedure of Tests.

The term "phenol value" is used in this paper to denote comparative values only. Although the conditions laid down in the standard Rideal-Walker technique were complied with as far as possible, no rigid adherence to this method is claimed. Hence the results here recorded of a series of comparisons against a phenol standard may be regarded as comparative values against phenol of unit value, but only as approximations to the strict bacteriological phenol coefficients.

The test organisms used were 24-hour cultures of *Staphylococcus aureus* and *Bacillus coli,* incubated and grown in nutrient broth at 37° C.

The broth, as used, was prepared by boiling minced cow's heart (from which the fat and fibrous tissue had been previously removed) in distilled water in the proportion of 1 lb. meat to 1,000 ml. water. After 20 min. boiling, the volume was made up to 1,000 ml., filtered, and 10 g. A.R. sodium

chloride and 10 g. pure peptone added. The mixture was again boiled for 20 min., filtered, and adjusted to pH 7·0 to 7·2 with N/10 sodium hydroxide solution. The phenol used for standard comparison had a congealing point of not less than 40° C., and was used in a 5 per cent. solution, being frequently standardised with N/10 bromine. Rimless tubes were employed. To facilitate flaming, lipped Pyrex tubes were used as seeding tubes in some cases. Transfers were made with a 4 mm. (inside diameter) single loop of medium gauge platinum wire. 5 ml. each of the stock dilutions were used per tube. These tubes were placed on the water-bath for 5 min. to bring to the required temperature of 20° C.; 0·5 ml. of the test culture was then added to each of the dilutions at definite time intervals. The culture was added from a graduated pipette holding sufficient material to seed all the tubes in any one set. Unfiltered culture was used, but it was thoroughly shaken 15 min. before use and allowed to settle. To ensure uniformity of conditions during the experiment it too was maintained on the water-bath at a temperature of 20° C.

The medication tubes were agitated, gently but thoroughly, just after the addition of the culture to ensure even distribution. Five minutes after the time of seeding, one loopful of the mixture of culture and diluted liquid was transferred to the appropriate sub-culture tube. At the end of the appropriate time-intervals the same procedure was followed with the second and succeeding medication tubes. Five minutes after the time of making the first transfer a second set of transfers was begun for the 10 min. period, and finally repeated for the 15 min. period. In the later tests, the time intervals were reduced to 2½ min., 5 min., and 7½ min., respectively. The effectiveness of the disinfectant was measured by examination of the sub-culture tubes for signs of growth after incubation for 24–48 hours at 37° C.

Preliminary Tests of Method.

In order to test the comparative results obtained from the technique outlined above and those from the standard test, it was decided to investigate certain substances of known Rideal-Walker coefficient. Tests were carried out against standard phenol solutions with lysol as the test substance. This lysol is stated by Martindale (5) to contain at least 50 per cent. of cresol. According to Hewlett and McIntosh (6) it has a Rideal-Walker coefficient of 2·5, and this figure is claimed for it also by the manufacturers. Details of the media and manipulation employed in this series of tests are here recorded:—

Composition of medium	Broth as described earlier.
"Acidity" of medium	pH 7·2.
Amount of culture medium per tube	10 ml.
Amount of culture added to diluted disinfectant	0·5 ml.
Amount of diluted disinfectant in tube	5 ml.

Condition of tubes in test	...	Plugged with cotton.
Temperature of tubes ...	...	20° C.
Time intervals	...	5, 10 and 15 minutes.
Size of transfer loop ...	...	4 mm. diam. (No. 27 Imperial guage).
Calculation of phenol value	...	Highest dilution killing in 10 but not in 5 min., divided by same for phenol.

The average phenol value obtained for lysol by the above method was 1·8.

Preparation of Emulsions.

The oils from the peat-tar distillation had to be emulsified to enable tests to be carried out. After some experiments with oils derived from a mixed coal-peat tar the following was considered the most effective emulsion:—50 parts of phenolic fractions; 20 parts of soft soap; 50 parts of 10 per cent. solution of A.R. sodium carbonate. The mixture was refluxed for 1 hour.

This emulsion passed the standard tests of solubility on dilution:—Two dilutions were prepared: the first containing 1 ml. of disinfectant in 5 ml. of water; the second was that required as a test of purity for higher Liquor Cresolis Saponatus (7) in which 5 ml. of disinfectant was mixed with 95 ml. of water. If no separation appeared in either case after standing for 4 hours, the disinfectant was passed for testing.

Examination for Phenol Value.

Two emulsions were prepared according to the specifications above: the first from the fractions boiling from 120° C.–210° C., and the second from those oils with a boiling point between 210° C.–300° C. For convenience these preparations were labelled P and Q, respectively. The following figures show the calculated phenol values of the emulsions from tests against a culture of *Staphylococcus aureus* resistant to phenol of dilution 1–90 (using time intervals of 5 min., 10 min., and 15 min.):—

Preparation P	...	Phenol value	9·3
Preparation Q	...	Phenol value	11·5

The above figures were only very slightly lower than the values obtained when *B. coli* was employed as test organism:—

Preparation P	...	Phenol value	9·8
Preparation Q	...	Phenol value	12·8

It appeared that the higher fractions were more potent germicides. A further distillation of the oils was effected, and the small quantity boiling above 250° C. was collected and emulsified as above. This third preparation (labelled R) was tested against *Staphylococcus aureus* to give the phenol value of 14·0.

The peat-tar fractions boiling between 120° C.–300° C. were now placed in a Claisen flask. Glass wool was packed tightly into the flask, which was

evacuated to a pressure of 20 mm. The flask was immersed in an oil-bath which was heated gradually, and the phenols were distilled. The first fractions, which came over in small quantity from 60°–70° C., were colourless oils, which were emulsified and tested as above, using however the time intervals 2½ min., 5 min., and 7½ min. In this case the phenol value was calculated by dividing the highest dilution which shows life in 2½ min. and 5 min., but not thereafter by the same for phenol. This emulsion (preparation S) gave a phenol value of 2·2, against *Staphylococcus aureus.*

Preparations P and Q were also tested under these time intervals, and gave values of 12·2 and 13·6, respectively, also against *S. aureus.*

A blank test was now run on the emulsifying agents used in all the above tests (20 parts soft soap and 50 parts of 10 per cent. solution of A.R. sodium carbonate). Under both time-interval systems the phenol value was found to be in the region of 0·5. (The method of testing used is, perhaps, less accurate with substances of low disinfectant powers).

The following table gives the complete list of phenol values for the various preparations, the figures for the time-intervals 5, 10, 15 min. being in column A and those for 2½, 5, 7½ min. in column B:—

Table 1.

	Against *S. aureus.*		Against *B. Coli.*
	A	B	A
Preparation P	9·3	12·2	9·8
Preparation Q	11·5	13·6	12·8
Preparation R	14·0	14·0	—
Preparation S	—	2·2	—
Emulsifying Mixture	0·5	0·5	—

4-triazo 3, 5-dimethylpyrazole aromatic hydroxyl reactions.

The substance 4-triazo 3, 5-dimethylpyrazole has been shown to develop characteristic and sensitive colour reactions with aromatic hydroxyl compounds (8). Some tests were carried out with this substance on the peat-tar oils used in the preparation of the above preparations with a view to comparing the probable constitution with the apparent phenol values.

Preparation of 4-triazo 3, 5-dimethylpyrazole.

5 g. of dimethylpyrazole prepared from acetylacetone and hydrazine sulphate were heated with 2 ml. of sulphuric acid (d. 1·8) and 4 ml. of fuming nitric acid (d. 1·5), the pyrazole compound being added slowly, and the reaction mixture meanwhile cooled in ice. The solution was then warmed on a water-bath for two hours, and the product poured on to crushed ice. Sodium hydroxide was added until the solution was nearly neutralized, when

4-nitro 3, 5-dimethylpyrazole separated as a thick white precipitate. This was washed with ice-cold water, and then dissolved in 5 ml. of water and 10 ml. of concentrated hydrochloric acid. 5 g. of tin were added gradually, the reaction mixture being immersed in crushed ice. The solution was then heated on a water-bath for two hours, and the tin precipitated as sulphide, after dilution of the mixture. Concentration of the filtrate gave the compound 4-amino 3, 5-dimethylpyrazole in the form of its hydrochloride.

This solution was diazotised with sodium nitrite in the cold (1·2 mols. hydrochloride to 2 mols. nitrite). The excess of nitrite was removed with urea, and 1·5 mols. of sodium azide were added in the cold. After standing for 3 min. sodium carbonate solution was added, and the triazo-pyrazole separated as a bulky white precipitate. It was filtered and washed with cold water, and used without further treatment in the following tests. Small quantities of the peat-tar phenolic fractions were placed in white dishes, and dissolved in dilute alkali. On addition of a small quantity of the triazo reagent to each dish, colorations developed slowly in each case. The following observations were made:—

TABLE 2.

Colour reactions with Triazopyrazole.

Samples.	After 30 min.	After 2 hours.	After 24 hours.
P	Light reddish-purple developed round the edges and slowly enlarged.	Deep purple throughout.	Very deep purple, almost dark blue.
Q	Reddish-violet round edges.	Reddish-purple throughout.	Reddish-purple darkening.
R	Reddish-brown streaks.	Dark blue.	Very deep reddish-purple.
S	No coloration.	—	—
Phenol	Bright-blue streaks, purplish at edges.	Deep blue throughout, faintly red at edges.	Very dark-red with bluish tinge.
O-Cresol	Bluish-violet.	Dark red.	Reddish-brown.
M-Cresol	Intense Blue.	Reddish-brown.	Dark brown.

The fractions from the peat-tar oils differ from the lighter hydroxyl constituents of coal-tar, and give mixed colours. In comparative tests the differences were quite distinct, although no quantitative test could be made. It is probable that these peat-tar oils have a much more complex structure embodying a wide range of phenolic substances.

Some Further Tests.

A number of emulsions on similar lines to the above were prepared from oils distilled from a mixed coal-peat tar produced in commercial gas-work carbonisation plants.

A series of twelve tests was performed on these oils, and phenol values ranging from 3·1 to 5·1 were obtained. Further to this, a number of experiments on the partial cracking of these oils were carried out and some interesting results noted.

Cracking of the Oils.

(*a*) *Vapour Phase.*—It was decided to endeavour to effect thermal decomposition without the application of pressure. The first attempts were carried out in the vapour phase. 200 ml. of the coal-peat tar fractions boiling between 0°–250° C. were placed in a distillation flask, the outlet of which was connected to an iron tube 97 cm. long and 1·8 cm. internal diameter. This tube was filled with unglazed porcelain chippings, and joined to a copper coil immersed in a water jacket. The outlet end of the coil was connected to a pyrex condenser. The pipe was heated to a temperature of 250° C. (measured by means of a thermocouple) throughout its length by a series of gas-jets. The oils in the distillation flask were boiled (208° C.), and the vapour passed through the tube. The distillate was collected in a 20 per cent. solution of sodium hydroxide, the alkali-soluble fractions being later salted out. In all, about 170 ml. of oils passed through the pipe and yielded 98 ml. of alkali-soluble distillate. The boiling point of the distillate was 187°–189° C.

Two separate disinfectant preparations were now made up according to the specifications already given for the uncontaminated peat-tar oils. The first, prepared from the uncontaminated coal-peat tar phenols, was called Preparation A, and the second, prepared from the same oils after they had undergone thermal decomposition, was called Preparation A_1. The phenol values of these emulsions were now investigated against staphylococcus and over 5, 10, and 15 minute intervals, and the following figures were obtained from a series of four complete experiments. It will be noted that the phenol values were not constant throughout, nor did they show a constant increase from A to A_1, owing to the varying amounts of coal-tar in the different original tar samples, but in all cases the figures for A_1 were higher than those for A.

Experiment No.	Phenol value of A.	Phenol value of A_1.
1	4·3	7·4
2	6·2	11·8
3	5·2	7·0
4	5·1	7·2

(*b*) *Liquid Phase.*—It was now endeavoured to affect a similar molecular rearrangement in the liquid phase. In this instance the oils were dropped slowly from a funnel on to an iron plate heated from 350°–380° C., and the vapour condensed and collected in alkaline solution, from which the phenols were extracted as before. This process gave a 77 per cent. yield of alkali-soluble tar oils which boiled at 204° C. A preparation of these oils (emulsified in the

same way as before) was labelled A_2. Various samples so prepared were placed in comparison with corresponding samples of A and A_1 (against *Staphylococcus aureus* and over 5, 10, 15 minute intervals). The results are tabulated below.

Test No.	Phenol value of A.	Phenol value of A_1.	Phenol value of A_2.
1	5·6	7·2	5·4
2	4·8	6·8	5·2
3	4·8	6·4	5·4

Similar experiments to the above conducted with uncontaminated peat-tar oils did not provide similar results, but further work is in progress on this aspect of the problem.

Summary.

Peat on distillation at 300°–350° C. yielded approximately 8 per cent. of peat-tar. The tar was fractionated, and the various fractions extracted with alkali. The extracts emulsified readily, and in preliminary tests of approximate bactericidal power gave high values when compared with phenol and lysol preparations. From qualitative colour tests with 4-triazo 3, 5-dimethylpyrazole, there is indication that substances other than simple phenols or cresols are present. Further work is in progress in the preparation of emulsions of higher tar-oil contents.

Cracking experiments to increase the activity of the oils give positive values with the mixed coal-peat tar fractions, but so far this treatment has not improved the peat-tar oils.

These preliminary experiments suggest the need for further experiments on the bacteriological examination of peat-tar oils prepared under different conditions.

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REFERENCES.

1. Morgan and Scharf.—Econ. Proc. Roy. Dubl. Soc., **2**, 10, p. 161 (1915).
2. Reilly and Kelly.—"Irish Peat Waxes," Stationery Office, Dublin (1943).
3. Donnelly and Reilly.—Sci. Proc. Roy. Dubl. Soc., **19**, 29, p. 365 (1930).
4. Davis.—"Peat in Michigan," Michigan (1907).
5. Martindale.—"The Extra Pharmacopocia," London, p. 926 (1928).
6. Hewlett and McIntosh.—"Manual of Bacteriology," London, p. 715 (1932).
7. British Pharmacopocia, London, p. 137 (1932).
8. Morgan and Reilly.—Trans. Chem. Soc., **105**, p. 442 (1914).

No. 25.

STUDIES IN PEAT.

Part 12.

MONA WAX (IRISH PEAT WAX) AND EMULSIFICATION.

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(Plate 10.)

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Irish peat containing as much as 45 per cent. of water can be extracted with petroleum-hydrocarbon solvents. Reilly and Emlyn (1) accounted for the extraction on the assumption that water-in-oil (W/O) emulsions are formed in the initial stages of the extraction, the water being consequently unable to prevent intimate contact of the solvent and the peat-wax. Finely divided carbonaceous matter in the peat was given as a possible emulsifying agent. It was also stated (*loc. cit.*) that very stable W/O emulsions could be formed with the molten wax as the continuous phase.

It has now been found that the wax extracted from peat by any of the solvents (e.g. petrol-alcohol, trichlorethylene, etc.) used in its production is capable, when present in the "oil" phase in relatively small amounts, of permitting water to be dispersed in certain hydrocarbon liquids (e.g. benzene, toluene, liquid paraffin). When the percentage of water dispersed in the oil is high (e.g. 75 per cent. of the total volume), the product has a consistency approaching that of a paste.

During the course of the latest experiments it was found that the products resulting from the dispersal of water in a wax-containing oil were always of yellow-brown colour, and they appeared to contain a voluminous precipitate. Examination under the microscope, however, showed them to be true emulsions. On being tested for type, these emulsions were found to be uniformly of the W/O variety. On standing in closed vessels, for periods up to eight weeks, these emulsions proved to be fairly permanent in the sense that no gross deterioration appeared to occur.

The peat-extract as a whole has, in previous papers, been designated "Mona wax," and this title will be used in subsequent description and reference.

The carbonaceous matter, originally contained in the peat, may have been mechanically collected in the extract, and may, perhaps, be suggested as the constituent which is effectual in the production of the wax-sponsored emulsions. Since, however, the percentage of this carbonaceous matter in the wax would, at most, be small, it is unlikely that the quantity of such carbonaceous material present, for example, in 0·075 gram of Mona wax, would sponsor an emulsion consisting of 60 ml. of distilled water dispersed in 20 ml. of an oil. It is possible, however, that during the initial stages of the extraction of moist peat by petroleum-hydrocarbon solvents, traces of carbonaceous matter in the ground peat may be responsible for the emulsification in the oil of "peat-moisture," thus allowing more intimate contact of the solvent and the wax-material. If this phenomenon were to occur even "locally" within the mass of the ground peat, and were a small quantity of the wax, as a result, to be extracted by the solvent, this extracted wax would assist in the emulsification of some of the remaining water. Such a process would be in keeping with the fact that, under certain conditions, the wax is capable of maintaining fairly concentrated water-in-hydrocarbon emulsions of low wax/oil ratio. The removal of this moisture would, in turn, aid in the extraction of more of the wax which, accordingly, would assist in the emulsification of more of the water. The effect, therefore, would be cumulative.

Since traces of carbonaceous material cannot be held to be the only possible constituent of Mona wax responsible for the maintenance of concentrated W/O emulsions, other factors have to be considered. From a study of the literature it would appear that no systematic investigation of the emulsions sponsored by peat-wax has been attempted. It was therefore necessary to prepare such systems, and to make a study of some of their characteristics.

Emulsions of water in hydrocarbon-oils were prepared, using Mona wax as the emulsifying agent. The hydrocarbon-oils used were benzene, toluene, xylene, and liquid paraffin. On account of its low volatility, liquid paraffin was the oil most commonly used in the work undertaken. It was found possible to prepare emulsions containing as much as 79 per cent. of dispersed water. Downward creaming[1] was a feature of the emulsions of water-concentration of even 75 per cent. With such a high water-content, however, the separated oil-layer occupied but a small bulk. That creaming should have taken place at all in such a concentrated emulsion is accounted for by the irregularity of the water-globules both in size and (often) in shape. An emulsion of 79 per cent. water in 21 per cent. liquid paraffin, with Mona wax as emulsifying agent, showed no evidence of creaming on being set aside for five weeks. Creamed emulsions were readily remixed by inverting the container a few times, allowing the stiff cream to run (when necessary), followed by gentle shaking of the container.

[1] The term "creaming" is used in the usual sense, viz., the separation, due to the influence of gravity, of a more concentrated emulsion from a dilute emulsion, i.e. the formation of two distinct layers, one consisting of the more concentrated emulsion, the other being the impoverished dilute emulsion (or even the clear external phase).

The emulsions, which had widely varying concentrations, were prepared by the intermittent-addition intermittent-shaking method (see later). It was observed on a number of occasions that when concentrated emulsions (75 per cent. water with liquid paraffin as the continuous medium and Mona wax as the "stabiliser") were allowed to stand for one hour, and then submitted to about thirty rapid continuous shakes, partial breaking of the emulsions resulted, bulk-quantities of water, estimated at from 5 per cent. to 20 per cent. of the total water in the system, being visible within the container. On setting these partially-broken emulsions aside for a short period, and then reshaking, most of the separated water disappeared.

Attempts made to homogenise water-in-liquid-paraffin emulsions by passing them through a hand-operated cream-maker resulted in partial breaking and partial homogenisation. On gently rubbing small samples of the concentrated emulsions the breaking of the emulsion was evident from the visible droplets of water which collected at intervals throughout the extended surface. More dilute water-in-liquid-paraffin emulsions (e.g. 40 per cent. water) failed to break when submitted to rapid continuous shaking, but the gentle rubbing of small samples again caused the appearance of small droplets of water. Emulsions of water in benzene and in toluene with the same emulsifying agent failed to break, irrespective of concentration, when subjected to rapid continuous agitation.

Until recently, the term "stability," as applied to emulsion-systems, lacked a sufficiently rigid significance. In 1939 King and Mukherjee (2), who then worked with soap-stabilised emulsions, defined the "stability factor" of an O/W system as the reciprocal of the rate of change (with respect to time) of interfacial area, per unit area of initial emulsion interface. In this connection the term "specific surface" ("specific interface," "specific interfacial area") may be expressed as the number of square decimetres of interfacial area per gram of dispersed oil. The authors assumed that the rate of change of the specific surface of an O/W emulsion is proportional to the initial specific surface (although in the subsequent work (3) they found, in some cases, that there appeared to be two rates—an initial rapid change, followed by a slower change), i.e., according to the simpler assumption,

$$-\frac{ds}{dt} = k_1 s_1 = \frac{s_1}{k},$$

where s = specific surface; s_1 = initial specific surface; t = time; k_1 = instability factor; k = stability factor.

They developed a method for the evaluation of k, based on size-frequency analyses, and were also able to compare the efficiencies of certain emulsifying agents when the concentrations of the agents were not too small. The work was later extended (3) to cases of emulsions of the O/W type stabilised by other hydrophilic colloids. As part of the analysis technique a representative sample of each given emulsion was suitably diluted, and the diluted sample

(where a soap was the emulsifying agent) was mixed with a little aqueous gelatine solution. One drop of the mixture was then placed on a microscope-slide, protected by the cover-glass, and the gel allowed to set (the gel was formed for the purpose of removing disturbances due to Brownian movement and to drift caused by convection, etc.).

The magnified image of the sample, thus treated, was projected on to a rigid screen of squared paper. This process enabled globule diameters to be measured. By choosing a statistically sufficient number of fields from a given sample, the total number of globules could be counted and arranged into size-groups.

Assuming that there were n_1 globules of mean diameter d_1, n_2 globules of mean diameter d_2, etc., the relative total volume, V, for all size-groups would be given by the expression $\Sigma n . \frac{\pi}{6} . d^3$, and the relative total surface area, A, by $\Sigma n . \pi . d^2$. The specific surface would then be given by $A/\rho V$ where ρ is the density of the dispersed liquid.

By such size-frequency analyses on a given batch of emulsion, after various suitable time-intervals, the value of the stability factor was calculated.

Lotzkar and Maclay (4) determined the comparative efficiencies of certain emulsfying agents by comparing the stabilities of O/W emulsions sponsored by these agents. The technique used by these workers involved the use of a hæmacytometer-slide. Specific surfaces were measured, from photomicrographs, in the same manner as in the previous case, but the formula used in calculating the stability-factor (k) was modified. These authors used the equation:

$$-\frac{ds}{dt} = k_1 s = \frac{s}{k}.$$

It was decided, in the present work, to investigate the "stabilities" of emulsion-systems in which liquid paraffin was the external phase, distilled water the dispersed liquid, and Mona wax the emulsifying agent. While it was appreciated that certain approximations would have to be made in the determination of globule-diameters (owing to the somewhat irregular shapes of some of the globules), an analysis on the lines already indicated was attempted.

A number of more dilute emulsions of water in liquid paraffin were prepared. One such emulsion, prepared by the intermittent-addition intermittent-shaking method, followed by a number of continuous shakes, consisted of 16 ml. of distilled water dispersed in 24 ml. of liquid paraffin in which 0·2 g. of Mona wax (extracted from peat by trichlorethylene) had already been dissolved. The emulsions thus prepared were fairly viscous. They were usually allowed to stand for one hour before being examined under the microscope. A drop of such an emulsion, examined under the low power without the use of a cover-glass, showed that the dispersion was somewhat

coarse. Fig. 1 is a photomicrograph of a representative field selected under the low power, of a sample of the emulsion to which particular reference has just been made, the sample being placed on a flat slide, and no cover-glass being used in the process. Fig. 2 is a photomicrograph showing the effect produced on the careful addition of a light cover-glass. It will be observed that some deterioration of the emulsion-sample has occurred. For more critical examination the emulsion was diluted with the continuous phase (liquid paraffin). A one-in-four dilution was found suitable for low-power work, while a one-in-eight dilution was suitable for high-power scrutiny. Under the high-power it was observed, in the cases of globules large enough to make such observation possible and when the upper surfaces of such globules were correctly in focus, that the globules were surrounded by films which had a cracked or wrinkled appearance (5). The films resembled "cellophane wrappings." Fig 3 is a photomicrograph showing some of the larger globules with the films in focus. No cover-glass was used in the high-power work just described. Even the most cautious subsequent addition of the cover-glass resulted in the breaking of these films and the coalescence of the water-globules in "pools." Minute scrutiny of the complete field revealed the presence of but few surviving globules. Samples of more viscous concentrated emulsions were tested for the effect of cover-glass addition. Breaking of the films also occurred in these cases, but to a minor degree. For certain other observations, use was made of a microscope-slide with round cavities.

Samples of emulsions of water in liquid paraffin, when examined immediately after preparation (i.e. without a rest-period) were found to contain globules nearly all of which were spherical in shape. While an emulsion under examination was still fresh, no cracked or wrinkled interfacial films could be observed. The films usually began to make an appearance when the emulsion had been resting for about 40 minutes.

A 40 per cent. emulsion of water in liquid paraffin, with Mona wax as the emulsifying agent, was prepared and was examined in the container, by unaided vision, at regular intervals, over periods of weeks. Apart from creaming, which was relatively rapid (as with most W/O emulsions), no gross change in the system could be observed. Samples of the same emulsion were subjected to microscopic examination at intervals over the same period. For this purpose the creamed material was gently re-mixed, representative samples withdrawn, and the samples placed gently on a slide. When a flat slide was used no cover-glass was added. When a slide with round cavities was employed, the sample added was insufficient to fill the cavity completely; a cover-glass could then be used. As a result of this microscopic examination no evident gross deterioration of the emulsion, over the period of examination, could be observed. Similar experiments with a duplicate batch of separately-prepared emulsion yielded similar results.

The breaking of these emulsions due to the addition, to samples on a flat slide, of a cover-glass, is further illustrated in the accompanying Figures (4

and 5). Fig. 4 is a photomicrograph of a representative field, taken under the high-power, of a diluted sample (1 in 8) of a 40 per cent. emulsion, aged 17 days, no cover-glass being used. Fig. 5 shows the deterioration which occurred on the careful addition of a cover-glass to the same sample.

At this stage of the work it became questionable as to whether the terms "stable" and "stability" could, even in the ordinary restricted sense, be applied to such a system. In cases where the dilute emulsion was set aside and subjected to no greater mechanical disturbance than the reasonably gentle re-mixing of creamed material, it was certainly established both by unaided vision and by microscopic examination that the system (apart from the inevitable creaming) showed good permanence for periods of weeks. The effect of the cover-glass addition, however, proves that the films which enveloped the globules could have had very little mechanical "strength" (6).

Dilution with liquid paraffin might have exerted some solvent action on the films which had already formed around the water-globules, thereby causing a certain amount of film-weakness. This was tested by diluting the emulsions with liquid paraffin containing the same percentage of dissolved Mona wax as that originally used in the preparation of the emulsion. No noticeable change in film-strength was observed as a result of this change in the nature of the diluent. These preliminary experiments in connection with the size-frequency analysis give some idea, independent of the question of "stability," of the nature of the films which enveloped the water-globules.

By taking, on a flat glass slide, a sample of a water-in-liquid-paraffin emulsion which contained about 40 per cent. of dispersed water, and adding the cover-glass, it was found possible, in some cases, by gentle pressure applied to the cover-glass by means of a needle, to obtain a field which contained globules large enough for the observation of some of the characteristics of the film. In some cases these globules seemed to be attached, through the agency of the film itself, to one of the glass surfaces. In other cases the globules were free to move. It was observed that, on touching the cover-glass, the film tended to change its shape and the enclosed water appeared to move, within the film, from one position to another. It was concluded that the film was somewhat sticky, and that the marks on the surface were wrinkles and not cracks. The adhesive characteristics of the film were further evidenced by the tendency, noticed in a number of instances, of smaller globules, already attached to larger ones, to remain thus attached.

During one particular examination of a water-in-liquid-paraffin system the possibility of the occurrence, in such systems, of emulsions of the "multiple" kind was demonstrated. One large film-covered globule contained, within itself, a number of apparently uniform smaller globules. Near the upper inner surface about five of these smaller globules could be counted, but on focussing below this level the smaller globules appeared to be very numerous and closely packed. Assuming that the large globule contained water as the "external" liquid, it could not be decided whether the smaller globules consisted of liquid paraffin surrounded by protective films (thus preventing

their coalescence) or of water surrounded by films (thus preventing inter-coalescence and mixing with the continuous water within the large membrane).

The process of film-rupture has been recorded by photomicrographs. Fig. 6 shows a globule in which the film has just broken at one side. Water (in focus) can be seen issuing through the broken part of the film. Fig. 7 shows a number of collapsed films (without water).

With regard to the permanence of the concentrated water-in-liquid-paraffin emulsion systems, it is likely that when the film is not subjected to undue mechanical stress it is sufficiently tenacious to prevent rapid coalescence of the water globules. In these cases the high viscosity of the system helps to prevent a too rapid impact of the globules when relatively gentle methods of re-mixing are employed. The rapid continuous shaking (involving violent contact with the inner surface of the container) was, however, vigorous enough to cause a serious amount of film rupture. It may be argued that it is a matter of hazard whether a given type and extent of shaking will, during the process of the making of the emulsion, succeed in emulsifying or de-emulsifying the given mixture. However, in no case did the intermittent-addition intermittent-shaking method used give any difficulty in the matter of the preparation of emulsions up to fairly high concentration limits (7), and in no case was the phenomenon of bulk phase-inversion experienced during the course of the preparation of the emulsions by this method. It may again be mentioned that interfacial films did not actually appear for some time after the emulsions had been prepared.

Whilst the above explanation of the permanence of concentrated water-in-liquid-paraffin emulsions seems reasonable, it must be admitted that no breaking occurred when more dilute emulsions of the same constituents were submitted to violent continuous shaking. It is possible that when the concentration of the water in the system is low, the film absorbed may be stronger. When benzene or toluene constituted the external liquid phase, violent continuous shaking of the system (dilute or concentrated) also failed to break the emulsion. Preliminary investigation of water-in-toluene emulsions indicated that the mechanical stability of such systems may be enhanced through an additional cause. These systems are receiving further attention by hanging-drop slide technique, etc. The effectiveness of various constituents of Mona waxes, and of treated Mona waxes, in emulsification processes, and of the effect of the presence, in the water, of selected electrolytes, are also being investigated.

Preparation of the emulsions.—Emulsions were usually prepared in 100-ml. Pyrex test tubes which were fitted with corks. The desired volume of oil was measured into the test tube, and a weighed quantity of the wax added. The wax was then dissolved by warming and gentle shaking. In some cases the inner walls of the tube were wetted with the solution thus made, but it was found that, in general, such a precaution was not essential for success in the preparation of these W/O emulsions. The solution was then brought to an arbitrary temperature (40° C. when liquid paraffin was used) and maintained at or near this temperature during the preparation of the emulsion. The

addition of the distilled water was carried out intermittently, the shaking being also intermittent, as described below.

Addition and shaking.—For the emulsification of 16 ml. of distilled water in 24 ml. of liquid paraffin ($\rho = 0{\cdot}894/15{\cdot}5°$ C., $\eta = 252$ Redwood No. 1 seconds at the same temperature) containing Mona wax:—2 ml. of distilled water, at approximately 40° C., was added to the liquid paraffin solution of the wax, the tube corked, and the system submitted to three sets, each of three vigorous up-and-down shakes. After a short pause another 2 ml. of the warm distilled water was added, and the system submitted to similar shaking. A third 2 ml. was then added and the shaking process was repeated. The remainder of the water was then added in two 5-ml. portions, the tube and contents receiving the same shaking treatment after each 5-ml. addition as was received after each 2-ml. addition. After the final shake of this series the system was allowed to rest for 1 minute, and then given six additional up-and-down shakes, individually vigorous, but with a slight pause between successive shakes. The emulsion thus formed was then set aside to cool in the corked test tubes. In most cases a period of at least one hour of standing had elapsed before submitting the emulsion to tests.

For the emulsification of 60 ml. of water in 20 ml. of the liquid paraffin containing Mona wax:—A procedure similar to that indicated above was followed, the addition of the distilled water being made in five 2-ml. quantities followed by ten 5-ml. quantities.

Weights of wax used.—Experiments were performed using various weights of wax per given volume of liquid paraffin. Thus, for 20-ml. volumes, the following weights were used:—0·075 g., 0·1 g., 0·125 g., 0·15 g., 0·175 g., 0·2 g., 0·25 g., 0·3 g., 0·35 g. Qualitatively, all these weights of wax gave fairly good emulsions, but the best emulsion resulted from the use of 0·2 g. per 20 ml. of the oil. It was therefore decided to use this proportion (or an approximation to it, e.g. 0·2 g. in 24 ml.) in the more extended work.

Determination of Type.—Various methods were used, all of them being sometimes applied to samples of the same emulsion, e.g. the Dye Method (slowly spreading colour with specks of Sudan III), the Dilution Method (dilution with the liquid which forms the external phase), Clayton's Electrical Conductance Method (a blank being performed on the water used), Direction of the Creaming (used in the case of the more dilute emulsions).

Homogenisation.—A small, hand-operated cream-maker was employed. The apparatus was fitted into a rectangular water bath, the water of which could be heated electrically to any desired temperature. The "cream" outlet passed through the base of the bath.

Photomicrography.—Photomicrographs of representative fields were taken by means of a simplified apparatus consisting of a microscope and a camera. The microscope was fitted with a Leitz × 12 Periplan ocular. A 1/6″ Watson Panchromatic objective was employed for high-power work, and a 1″ Swift objective for low-power work. The microscope-tube was extended to 155 mm. A normal miniature camera with a 5-cm. lens, operating on 35-mm. perforated

film, was used, the lens being set at the infinity mark. In addition to the 50-mm. extension due to the camera, there was an extension of 16 mm. due to a lens-hood attached to the camera. Slides were examined in a horizontal plane to minimise movement of globules, and when a suitable field had been chosen, the camera was clamped rigidly in position and the exposure made. The degree of linear magnification obtained on the film was 110 when the high-power objective was used, and 15·4 when the low-power objective was employed.

SUMMARY.

Mona wax acts as an emulsifying agent which sponsors the emulsification of water in hydrocarbon oil. Emulsions of water in liquid paraffin containing up to 79 per cent. of water have been prepared and examined. The mechanism by which moist peat is readily extracted by petroleum-hydrocarbon solvents has been accounted for in terms of the effectiveness with which the wax itself acts as an emulsifying agent.

The intermittent-addition intermittent-shaking method resulted in the satisfactory emulsification of water in the oils chosen.

The existence, in the case of emulsions of water in liquid paraffin (where Mona wax was used as emulsifying agent), of plastic-like, interfacial films, surrounding the water-globules, has been demonstrated. Such films have but little mechanical strength. A suggestion as to the mechanism of the breaking, by specified methods, of concentrated water-in-liquid-paraffin emulsions (sponsored by the wax), has been put forward.

While the emulsions were reasonably permanent, in the sense outlined, it is considered that any coefficient arrived at by the methods of size-frequency analysis (due precaution being taken against submission of the interfacial membrane to undue external mechanical disturbance) might, in the case of such systems, be termed a "factor of permanence" rather than a "stability factor."

DESCRIPTION OF PLATE 10.

FIG. 1.—Water-in-liquid-paraffin emulsion—Mona wax as emulsifying agent. Without cover-glass; × 42.

FIG. 2.—Effect produced on gentle addition of cover-glass to sample; × 42.

FIG. 3.—Interfacial films on emulsion globules; × 300.

FIG. 4.—Diluted sample of water-in-liquid-paraffin emulsion (aged 17 days); Without cover-glass; × 300.

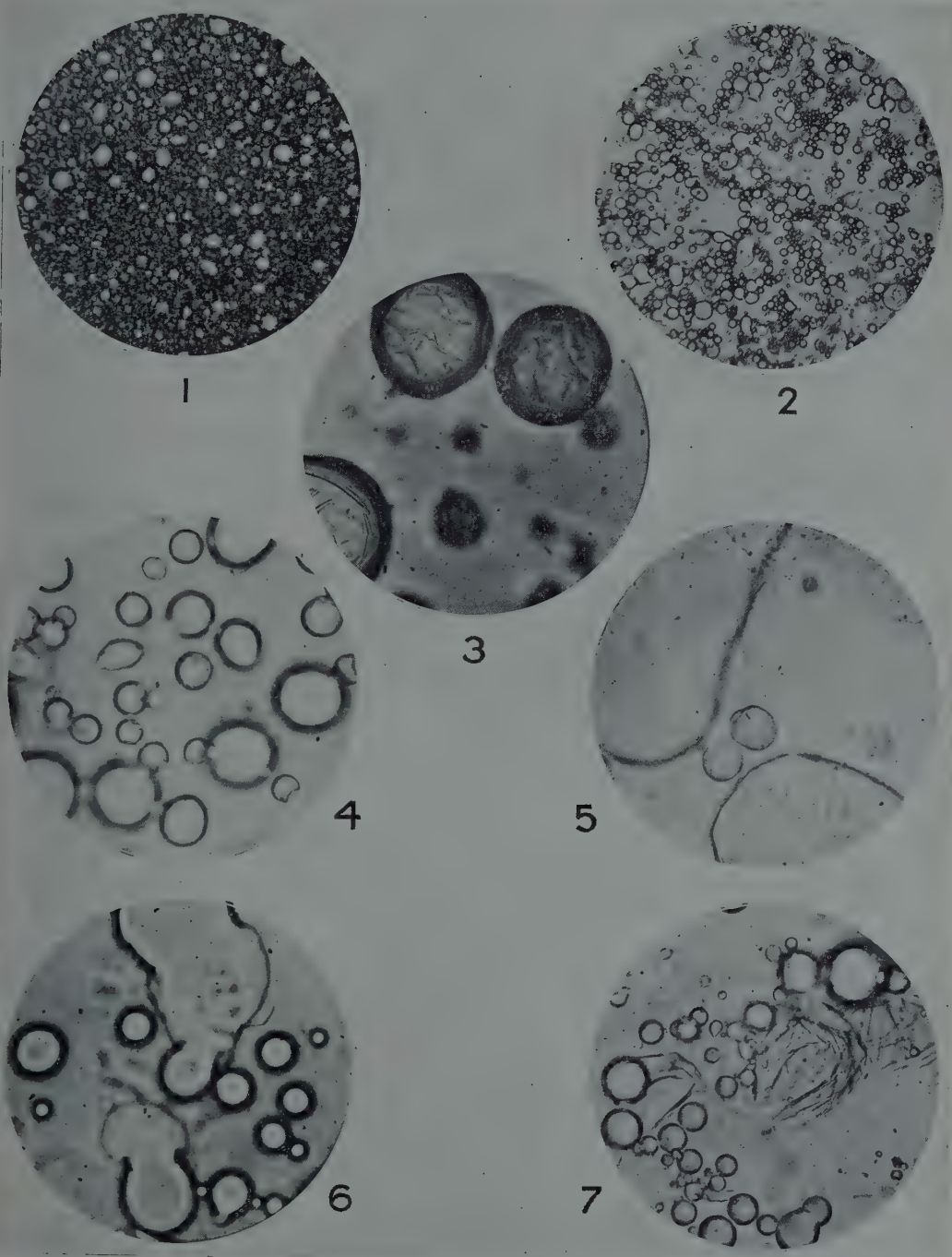
FIG. 5.—Effect of adding cover-glass to diluted sample; × 300.

FIG. 6.—Process of interfacial-film rupture. Issuing water in focus; × 300.

FIG. 7.—Collapsed films, without water; × 300.

REFERENCES.

(1) Reilly and Emlyn.—Sci. Proc. Roy. Dubl. Soc., 1940, **22** (N.S.), 267.
(2) King and Mukherjee.—J. Soc. Chem. Ind., 1939, **58**, 243.
(3) *Ibid.*, 1940, **59**, 185.
(4) Lotzkar and Maclay.—Ind. and Eng. Chem., 1943, **35**, 1294.
(5) In connection with the formation of adsorbed interfacial films, see, for example, Serrallach and Jones, Ind. and Eng. Chem., 1931, **23**, 1016, and on the strength of such films, *ibid.*, 1933, **25**, 816. Other references are given in "Emulsions and Their Technical Treatment" by Clayton, London, 1943.
(6) See discussion, by Pink, in a paper "The Stabilisation of Water-in-Oil Emulsions by Oil-Soluble Soaps," on the conditions to be fulfilled so that a precipitated soap may act as an emulsifying agent, Trans. Farad. Soc., 1941, **37**, 183.
(7) Cf. Briggs, J. Phys. Chem., 1920, **24**, 120; Sibree, Trans. Farad. Soc., 1930, **26**, 26, and *ibid.*, 1931, **27**, 161; Cheesman and King, *ibid.*, 1938, **34**, 594; Martin and Hermann, *ibid.*, 1941, **37**, 25.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No. 26.

FERTILIZATION AND EARLY EMBRYOGENY IN *PODOCARPUS ANDINUS*.

By W. J. LOOBY and J. DOYLE.

(Plates 11 to 14.)

[Read June 27. Published separately July 27, 1944.]

This paper continues one recently published (Looby and Doyle, 1944) in which an account was given of the gametophytes of *Podocarpus andinus* Poeppig up to fertilization. In that paper the interest of the relation of this species to other podocarps, the pertinent literature, the source of the material, and the like were described. Here the development is further followed to include fertilization, proembryo, and early embryogeny.

(A) *Fertilization.*—The structure of the mature archegonium and the nature of the two very unequal male cells are fully described in the earlier paper.

At maturity a discharge of cytoplasm takes place between the neck cells (Fig. 1), drawing the female nucleus up to a point, and forcing the male cells back to the top of the canal made by the downward passage of the pollen tube. The discharge area is shown more clearly in Fig. 2. Immediately above the dark discharged mass lies a layer of pollen tube cytoplasm in which a number of the sterile nuclei are still visible. This phenomenon seems unique in conifers. Owing to the definite granulate structure of the archegonial cytoplasm the remnants of the discharged material can be readily recognized in later stages. It is thus normal and not due to unsuitable pressure on turgid archegonia during the collecting.

The next stage available, Fig. 3, showed the female nucleus contracted back and the male nucleus, indicated by its dense nucleolus, passing between the neck cells. The main mass of the discharged cytoplasm in this case was to be seen in a nearby section of the ribbon. The functional male nucleus, with some at least of the male cytoplasm, comes to lie near the large archegonial nucleus, Fig. 4, and soon becomes partly embedded in it, Fig. 5. The large dark body in this upper male nucleus is its characteristic nucleolus. Fig. 61 shows the relations of these nuclei to the whole archegonium. The male nucleus coalesces with the female one, Fig. 6, the nucleolus disintegrating and the delimitation between the nuclei disappearing, thus leading to the typical fusion nucleus, Fig. 7. Fusion of two such nuclei as these offers a difficult cytological problem. Many other stages were available, but as the essential nature of the phenomenon cannot be explained these stages are omitted. The male cytoplasm which entered appears as the darker zone to the right of the male nucleus

in Fig. 4, above the fusing nuclei in Figs. 5 and 6, and to the left of the fusion nucleus in Fig. 7, but it never forms a special sheath. The second smaller male nucleus may be left outside, but it commonly enters; the small dark body above the fusing nuclei in Figs. 5 and 6 is its nucleolus. The sterile nuclei from the pollen tube also commonly enter, but they are ephemeral and their identification usually uncertain as nuclei from the jacket cells make their way into the top of the archegonium about this stage. They are shown in Fig. 8, the clump of tiny nuclei just above the large fusion nucleus of which only part is included.

The only reference to development in *Stachycarpus* is the short incomplete survey by Sinnott (1913) of some stages in *P. spicatus* and *P. ferrugineus*, but no data on fertilization are given. A drawing is given, for the second of these species, of a stage, said to be just before fertilization, which shows a pollen tube with male cells over an archegonium. Only a small nucleolus is drawn in the functioning male cell, and it is thus uncertain whether this male cell is still undeveloped or whether the enormous nucleolus of *P. andinus* is peculiar to this species. He also states that the male nucleus is about one half the size of the female nucleus. Actually in his drawing the diameter of the female nucleus is roughly four times that of the male, the former being thus about sixty-four times larger than the latter. The marked inequality in the size of these nuclei in *P. andinus* is evident from Figs. 4 and 5.

(B) *The proembryo.*—The first division of the fusion nucleus, which has been figured by Sinnott (1913) for *P. spicatus*, takes place in the upper part of the archegonium. The mitotic figure, minute and intranuclear, is shown with reference to the whole archegonium in Fig. 9, and in relation to the fusion nucleus in particular in Fig. 10. In both it is indicated by an arrow, the dark body above it being a protein body displaced from the cytoplasm in cutting. The problem of the relation of chromosome to resting nucleus is certainly presented here in an acute form! The two nuclei so formed slip out from the remains of the fusion nucleus, still visible at the top of Fig. 11, and pass some way to the middle of the archegonium, Fig. 11, in which position the second division takes place. The location of this division in relation to the whole archegonium is shown in Fig. 62, the divisions, simultaneous and again intranuclear, in Fig. 12. In this case the nuclear substance surrounding the mitoses rapidly disappears as such, and, even while still in metaphase, the mitoses may appear to lie merely in a slightly altered zone of cytoplasm (Fig. 13). Three of the resultant nuclei shortly after completion of telophase can be seen in Fig. 14 lying in a special zone of cytoplasm, the residue of the fusion nucleus being still visible above. The four nuclei, with a certain amount of cytoplasm surrounding them, continue to move towards the archegonial base and increase in size (Fig. 15). In the meantime the basal third, or less, of the archegonium becomes more compact than the upper part, which is loosely vacuolate and shows increasingly an appearance of marked senescence, somewhat as is figured in a later stage in Fig. 64. When the

proembryo nuclei reach the top of the denser basal zone they lie clumped together (Fig. 63), but soon slip free from any surrounding cytoplasm and pass cleanly into the basal area. Two of the nuclei are shown well down in this zone in Fig. 16. A further carving out of a still denser area of cytoplasm smaller than the first now takes place, as seen in Figs. 17 and 18. In the section from which Fig. 17 was taken this zone is cut a little tangentially but shows three of the nuclei, Fig. 18 showing the form of this final embryonic area better, although the nuclei, being in a deeper focus, are only indicated. In this small area the subsequent stages in the proembryo take place, and during most of this period, owing to the denseness of the cytoplasm and its great avidity for any dye, differential staining proved difficult. The size of this area relative to the whole archegonium is shown by the small black tip of the right-hand archegonium in Fig. 64.

Sinnott (1913) mentions the passage of four proembryo nuclei to the base of the archegonium in *P. spicatus* and *P. ferrugineus,* but his account of the subsequent stages is so brief and inadequate that no further reference need be made to it.

Three further successive simultaneous divisions now take place, resulting in 32 free nuclei before wall formation. The series of figures which follows to illustrate this is a selection from a much more complete series of stages, and is used to show that the development was examined fairly completely. The four mitoses of the third embryonic division are shown in Figs. 19 and 20. Figs. 21 to 23 are from one proembryo at the 8-nucleate stage, five nuclei being shown clearly and the other three more faintly, the remaining sections from this proembryo being omitted. Fig. 24, the same stage as Fig. 22, but at a lower magnification, shows the relations to the lower part of the archegonium. The mitoses of the fourth division are given in Figs. 25 and 26, parts of two mitoses appearing in each. Figs. 27 and 28 show two sections from another proembryo at this stage, but at late telophase, the small black dots being the chromosome clusters. Nine such clusters can clearly be seen, the other seven being equally clear in sections here omitted. The sixteen nuclei so formed enlarge, but remain scattered without obvious order, and show no sign of walls or cleavage between them. Fig. 29, in which nine nuclei can be counted, is from one section of a proembryo at this 16-nucleate stage. The fifth simultaneous division itself was, unfortunately, not found, but a stage very shortly after it was obtained. It might have been advisable to present a more complete series here, but, as a long series would be necessary to demonstrate the full number of nuclei and their organization, only a limited number of illustrations are included. One section from the youngest 32-nucleate stage available is shown in Fig. 30. Here the nuclei are small and obviously only recently formed, while some still tend to lie in pairs. These nuclei as they enlarge become organized with some regularity, but all the nuclei are not included in this organization. Fig. 31 is a rather tangential section of another slightly older stage in which the nuclei are already coming to lie in some order, and, above the functioning upper tier, at least four nuclei

can be seen lying loosely in the cytoplasm. These may be referred to as the *relict* nuclei. The number of the lower compacted nuclei is always less than 32, perhaps as low as 22. While it is not easy to count accurately a large number of nuclei in dense cytoplasm with poor differential staining, yet the total counts in the preparations available varied from 30 to 33 if the relict nuclei are included. These relict nuclei tend to disintegrate rapidly. Two can be seen above in Fig. 30, one of which is already large and faint while the other shows a premature mitosis. This latter appearance was frequently seen, but the mitosis is seldom, probably never, completed, the chromosomes breaking up to irregular dark masses. The number of relict nuclei varies roughly from 6 to 10, and thus the number of nuclei taking part in the proembryo proper must vary from about 22 to 26 or so.

The functioning nuclei become arranged in tiers, of which only the upper is commonly well defined. Figs. 32 and 33 (on the left; the group on the right is an 8-nucleate stage in the other archegonium) show stages in the arranging of the nuclei shortly before cleavage. It is clear from Fig 33 that the tip is occupied by a single nucleus. No membranes are formed on the spindles of the last division, and wall formation seems to be by cleavage planes. Material did not suffice to follow the cytology of this cleavage, which is sufficiently indicated for present purposes in Fig. 34. The form of the young proembryo, as shown in Figs. 35 and 38, is rapidly reached. The upper tier of nuclei have formed a group of elongating cells still open above, the others forming an embryo group of completely walled cells, a large terminal cell being very conspicuous. Differential staining is still uncertain, but the large nuclei of the upper tier and the nucleus of the cap cell can be seen in these two figures, while Fig. 36, another section from the same proembryo as Fig. 38, suffices to show that all the cells are still uninucleate. This section was obviously one tangential to the embryo, and the figure includes only embryo cells at a higher magnification, the edge of the cap cell being just indicated below.

The nuclei of the upper open tier divide more or less simultaneously to form the prosuspensor tier proper, and another tier of cells above open to the archegonium. This tier could be called the *open-cell* tier. An early stage in its formation is shown in Fig. 39, and one of the many later stages available in Fig. 41. This tier is not permanent, and may be quite ephemeral. At the same time, or perhaps a little later, the nuclei of the embryo group, with the probable exception of the terminal cell, divide, again more or less simultaneously, to give rise to the characteristic podocarpean feature—the binucleate condition of these proembryo cells. Fig. 40 shows these cells from Fig. 39 at a higher magnification. The focus is on the two upper cells, in which the mitotic figures are clear, but in all the cells when focussed successively some stage in division can be seen. The resulting binucleate condition of the cells is shown in many of the succeeding figures, notably 42, 43, and 51. In only one case were two nuclei definitely determined in the terminal cell. This condition may have been obscured in some cases owing to the denseness of the cytoplasm, but certainly in many cases only one nucleus was present. The nuclei in the

embryo are at first difficult to separate from the cytoplasm, but when this can be done they show the simple rather undifferentiated appearance recorded in Fig. 42, which should be compared with the very different nuclei of Fig. 43, a difference which will be explained shortly.

As already mentioned the number of nuclei and resulting cells in the proembryo may vary, and of these the numbers forming the embryo group and prosuspensor tier, respectively, may again vary within wide limits. Judging from the examination of about 36 proembryos before suspensor elongation the number in the upper tier may vary in general between 9 and 13, once being as low as 6 and once as high as 15. In most cases the number in the embryo group lies between 11 and 15, with an occasional 9 or 10 and once dropping as low as 7. The proembryo which had 6 prosuspensors had 23 or 24 in the embryo group, but this high figure was only found once, the next highest figure to it being 17, with 9 or 10 prosuspensors.

(C) *Early embryogeny.*—Reference to the dates given under "Explanation of Plates" shows that fertilization takes place about mid-June, and the stages so far described are completed in little over a week. Slow growth of the prosuspensors now begins, and the subsequent developments in the embryo cells, except for one small change, are not found till about mid-July when another bout of intense activity occurs which finally fashions the young embryo proper. About the time, however, that the prosuspensors first show coiling the embryo cells enter a peculiar phase. The cell contents tend to contract greatly in fixation, and the cytoplasm to stain even more intensely than before, so that the group of cells looks like a dark degenerating clump. Fig. 37 shows such a stage, which may be called the *resting* stage as it continues till elongation of the prosuspensors is almost complete. The cells gradually come out of the resting stage before the next phase supervenes; they fix better, the nuclei contrast more strongly with the cytoplasm, and nucleoli become very distinct. This is the appearance already referred to as shown in Fig. 43. The difference is quite obvious in most cases. The terminal cell begins to show signs of degeneration during the downward growth of the suspensor, and may be totally degenerate at the stage of Fig. 43, although its form may be retained for some time later, presumably due to the firmness of its wall. A couple of the embryo cells above it may occasionally degenerate also. It may be that this specialized cell facilitates the penetration of the embryo into the starch cone of the endosperm. Only in two other species in all the podocarps, and these also species of *Stachycarpus*—viz. *P. spicatus* and *P. ferrugineus*—does a similar cell seem to occur. In the former it also degenerates, but its fate in the latter is uncertain (Buchholz, 1936). In both cases it is described as containing two nuclei like those in the other embryo cells. A rechecking of this might be advisible.

From about this stage onwards embryos were dissected out whole as well as sectioned. As no cleavage took place later the dissected ones added little except confirmation to the knowledge gained from sectioning. A couple of photographs of dissected embryos are included. Fig 44 shows one photo-

graphed whole at the stage already shown in Fig. 43, although naturally nuclear detail is not obvious without special staining and special photographic technique.

In later embryos of podocarps the cells are known to be uninucleate. Buchholz (1936) suggested for *P. spicatus* that "cell plates appear between the two nuclei of each embryo initial or unit followed by other cell divisions," but no figures are given. Doyle and Looby (1939) made a similar statement for *Saxegothaea,* and supported it with a published figure. But material about this stage in *Saxegothaea* was scanty, and this figure was taken from *one* dissected embryo only, re-examination of which showed that the appearance might be open to other interpretation. In any case it is unsafe to draw conclusions from a single preparation, so particular attention was given to this stage in *P. andinus.* When this work was well advanced another paper by Buchholz (1941) came to hand. In this paper he figured a double division in *one* of the two embryo initials in *one* proembryo in *P. totarra,* the other embryo initial having become four-celled. The further statement is made that "no doubt the wall between two binucleate cells is delayed until after this pair of spindles has given rise to four nuclei." The meaning of this is not clear and perhaps confuses a further important statement in which he goes on to suggest that it is probable that in all podocarps the binucleate proembryo cells *directly* form four-celled groups. Actually this latter interpretation, put by Buchholz on his apparently isolated observation in *P. totarra,* is probably correct, as it is the condition also shown in *P. andinus.* The problem in this type is, however, a somewhat more difficult one. The point to be settled is whether a membrane forms between the two nuclei of a binucleate proembryo cell before the nuclei divide, or whether a simultaneous division of the two nuclei leads through a four-nucleate stage to a four-celled grouping. In *P. andinus* it is not a matter of following one or two embryo cells, but a group of cells very variable in number. An embryo with a large number of cells could be in quite a young stage or in an older stage in which divisions had taken place; and in whole dissected embryos in which divisions could be seen it was impossible, owing to the thickness of the embryo groups, to be satisfied with the location of the mitoses. Sectioned embryos, however, gave good results.

This phase—the change from the binucleate to the uninucleate condition of proembryo cells—is, in *P. andinus,* passed through rapidly after the embryo group recovers from the resting stage, and misinterpretation was fatally easy. At a certain stage in development, which may be called the *tetrad* stage, most of the embryo clump is seen to be composed of uninucleate cells compacted in four-celled groups. In each of the Figs. 52–54, which are sections from the same proembryo, at least one such group is obvious, these being the tetrads lying in the plane of the sections. The other tetrads are cut at various angles. Thus the cells grouped at *a* in Fig. 52 are probably not a single tetrad, but parts of two different ones. This tetrad stage could readily be recognized in dissected embryos, but was difficult to photograph, again owing to the thickness

of the whole embryo. In Fig. 56 the arrow points to a fairly distinct one, and in Fig. 57, from a partly crushed embryo, two tetrad groups can be picked out. Further, however, stages such as those in Figs. 45 and 46 were common about this phase. The first obvious interpretation of Fig. 45, showing two simultaneous mitoses in contiguous cells (flanked on the left by a binucleate cell), is a derivation from a binucleate cell in which a wall first formed between the nuclei followed by a division in each resulting cell, thus giving a tetrad. The lower group of cells in Fig. 46, from the edge of the *same* proembryo as Figs. 52–54, could be a similar case in which one of the cell pair, formed by the first membrane, lagged in its division a little behind the other. These are, however, in reality *post*-tetrad divisions.

A typical example of what actually occurs in *P. andinus* is shown in Figs. 47–51. These are serial sections from the same embryo, obviously in a young stage as some of the cells are still binucleate, Fig. 51. Fig. 47 shows on the right a cell in which two simultaneous divisions, indicated by the arrows, are taking place. The mitotic figures are not in the same plane, so only a little of the right-hand group of chromosomes appears. Figs. 48 and 49 are views of the next section in two different focal planes. The focus in Fig. 48 is on the fragments of the left mitosis in Fig. 47, the mitosis on the right being now more prominent. In Fig. 49 this division on the right is focussed, that on the left, owing to the lie of the blurred chromosomes in relation to the edge of the cell, giving the appearance of a faint nucleus. To the left above is a cell with a large nucleus in early prophase. A second nucleus in this cell appears in Fig. 50, which also shows a clear single division at the lower right. In Fig. 51, in the same position, another mitosis can be seen. These two divisions are almost certainly in the one cell, but when divisions are in different sections, as here, it is hardly possible to *prove* that they are in the same cell, still less to determine whether or not a membrane exists between them. Other, perhaps better, cells might have been selected instead of this series, but it seemed of greater interest to show what an average one of the smaller embryos actually looks like on the slide when completely sectioned.

From such double divisions the tetrads are formed, and in each of the Figs. 47–50 some stage in a tetrad grouping, much out of focus, can be seen. It is obviously of interest to determine how the membranes are formed between the resulting four nuclei, and the relations of these membranes to the old cell wall, but this has not yet been done. All the material available at stages such as these has been stained for chromosomes and nuclei. A further intense collecting and a restaining for pectic membranes will be necessary for a proper cytological examination, but a further reference to Figs. 54, 55, and 52 is of interest. The cell marked with an arrow in Figs. 54 and 55 (to the right of the very distinct tetrad) is also drawn in outline in Fig. 59. This cell is one shortly after the double division, but before membrane formation. It suggests that the pairs of nuclei are first separated by a cleavage plane in the position in which a membrane has been claimed to occur in *Saxegothaea* before division. Now in the one cell drawn and described by Buchholz (1941)

only one spindle lies in the plane of the figure, the other is almost at right angles to this plane and only an indefinite clump of chromosomes is shown, but a membrane is drawn developing across the single spindle visible. Of course such membranes, even if present between the nuclear pairs in Fig. 59, would not be visible owing to the lie of the cell, but the four nuclei are all in an advanced resting stage, and no trace of any spindle on which a membrane would develop could be detected either here or in any other case of a paired mitosis. It is possible that a cleavage membrane is formed here also. In the cell marked with an arrow in Fig. 52 and outlined in Fig. 58 four nuclei are present, and membrane formation is definitely in progress, but no spindle connections could be made out between the nuclei. However, until a large number of suitably stained intermediate stages are available, the question of membrane formation must remain uncertain.

In view of these data from *P. andinus* it is clear that, not only is Buchholz's (1941) interpretation of his single fortunate observation correct, but it is probable that this tetrad stage is fundamental in *Podocarpus,* since it occurs in one of the simplest forms with a large group of embryo cells, and in one of the most advanced species with only one or two such cells in the proembryo. Buchholz's older figure (1936, Fig. 9) probably shows this tetrad stage also in *P. spicatus.* It will be important to examine this stage in other genera. Older figures, again of Buchholz's (1933, Figs. 3, 7), almost certainly show this in *Dacrydium cupressinum* as well. Early revision of *Saxegothaea* is clearly essential. The tetrad stage may well prove an important family character like the binucleate condition in the young proembryo.

During the period of tetrad formation the cells of the embryo group enlarge a little, the whole group becoming looser and more bulged, as a comparison of Figs. 53 and 56 with Fig. 44 shows. There is, however, no embryo cleavage. Although it is easy to visualize how an increased independence of these tetrads could lead to the cleavage polyembryony of *Dacrydium* and some species of *Podocarpus,* yet in *P. andinus,* although hundreds of embryos at various stages were examined, not the faintest suggestion of cleavage or lobing was seen.

On completion of the tetrad stage divisions take place in the now uninucleate cells, the whole embryo becoming gradually more compact. Fig. 60, from a dissected embryo, shows a typical example just before the formation of embryonal tubes. For a short time, however, there seems to be a tendency for neighbouring cells derived from the same tetrad to divide more or less together, the divisions in Figs. 45 and 46 being of this type. As a result the developing embryo may show temporary tetrad-like groupings, or may appear as if faintly divided into sections, but the change from tetrad organization to that of a homogeneous embryo system is rapid. Figs. 69 and 70 are typical embryos with developing embryonal tubes. In the latter figure the tubes have only recently begun to elongate and are just pushing up the prosuspensors; in the former they are clearly much further developed. In both, the original terminal cap cell, which never enters into the organization of the embryo proper, can still be seen as a small pointed dark body at the tip.

The fleshy fruits, about the size of a cherry and pleasant to the taste, fall before the embryo is mature. On the oldest embryo met with at this stage the first development of cotyledons was just discernible. The same early fall was recorded for *Saxegothaea* (Doyle and Looby, 1939). This may be due to the trees growing outside their proper range, but Buchholz (1936) mentions a similar condition in seeds of *P. ferrugineus* gathered in New Zealand. It may be that an after-development of the embryo is another podocarpean feature. The phenomenon is worth closer investigation.

The endosperm in the meantime has been developing in a manner essentially similar to that already described for *Saxegothaea* (Doyle and Looby, 1939, Figs. 2c, d; pp. 131–2). The general appearance at a low magnification is shown here in Fig. 68 for comparison. The embryo in this section is that shown in Fig. 70.

The prosuspensor length varies considerably but averages about 6 mm.

(D) *Variations in embryogeny.*—These were strikingly few. Fig. 67 illustrates a condition found twice. This is a young proembryo in which the upper open-cell tier has not yet been cut off, and in which the embryo cells are still uninucleate. There are, however, at least 45 cells in the embryo group, 23 can be counted in this one section. These cases are almost certainly derived from an early stage in which a further free nuclear division took place before wall formation, giving 64 nuclei in all, which could clearly yield about 45 embryo cells, about 10 in an upper open tier, and a few relict nuclei. Rosette cells, at least in the sense used by Buchholz, were rare, appearing in not more than six embryos in four of which there were only one or two (Fig. 65). Fig. 66 is a diagram of a section from a type found twice. In view of the emphasis laid by Buchholz on the so-called rosette cells, such as those in Fig. 65, and of the fact that the cutting-off from the upper proembryo tier of a special open-cell tier seems an important phase in early podocarpean embryogeny, the origin of these smaller walled cells should be determined. That can hardly be expected from so stable a type as *P. andinus,* but may be attempted in some more variable species. It is possible that later divisions may occur in the prosuspensors, as one of these in the embryo from which Fig. 67 was taken showed a clear mitosis in metaphase.

(E) *Remarks.*—Any full discussion on development in the podocarps as a whole is postponed until work, at present in progress on *P. nivalis,* is completed, but a few points need further short comment here.

1. In only one other species from any genus of podocarps has the development of the proembryo and the early embryogeny been reported. This seems to have been done for *Podocarpus nagi* in a paper by Tahara (1941) which was not available and is only known from a short reference by Buchholz (1941). How much detail is presented in the paper or what wealth of material was available does therefore not appear. However, the proembryo is described as having 32 free nuclei before wall formation. These nuclei become organized into a single lower tier of 7–9 walled embryo cells and an upper tier of 23–25 cells open to the archegonium above. The upper tier is

described as forming, by more or less simultaneous division as in *P. andinus,* a prosuspensor tier proper and an open-cell tier which is not permanent. Whether or not true relict nuclei are extruded is not stated. Now in most cases the number of free nuclei that form the early proembryo in podocarps is not known accurately, and is judged from the number of prosuspensors and embryo cells that can be counted in older stages from dissected out embryos with the prosuspensors well grown. The numbers obtained are variable, but in most podocarps the number is probably sixteen. No proembryo has been accurately followed from the 16-nucleate stage, so that it is not known if any of the nuclei in such cases are extruded. Extrusion of a few nuclei might account for some of the variations in the numbers recorded, but in only four other species, in addition to the definite cases of *P. andinus* and *P. nagi,* is a 32-nucleate stage probable, judging from the large number of prosuspensors present. These species are *P. usumbarensis, P. gracilior, P. nankoensis* and possibly *P. blumei* (Buchholz, 1941, Tahara, 1941). It is of interest then that three of these species, *P. andinus, usumbarensis* and *gracilior,* belong to the sub-genus *Stachycarpus,* that the other three all belong to the section Nageia, and that, of these latter, two of them, *P. nagi* and *nankoensis,* belong to that sub-division of the section in which no fleshy receptacle is formed, the ovular arrangement resembling that in *Stachycarpus.*

2. The formation of an upper *open-cell tier* in *P. nagi* is also important. Coker (1902) suggested that such a tier was formed in the one species of Eupodocarpus studied by him, but had not enough evidence to be certain. Now that that stage is known to occur re-examination of Coker's figures clearly shows that such a tier was developed. Further in one of Buchholz's papers (1941) a figure is given (his Fig. 1) of a stage in *P. dacrydioides* with elongating prosuspensors above which are drawn a number of nuclei, referred to by him merely as relict nuclei, which must have had a similar origin. Thus in four sub-divisions of *Podocarpus*—the sub-genus *Stachycarpus* and the sections Nageia, Dacrycarpus and Eupodocarpus—the upper tier of embryonic nuclei divide to give an *open-cell tier* above the functioning prosuspensors. This condition must be considered just as fundamental in podocarpean embryogeny as the binucleate embryo cells proper. Thus immediately after primary membrane formation all the nuclei divide, roughly simultaneously, those below to form the binucleate cells of the embryo, and those above the uninucleate prosuspensor and open-cell tiers, the latter not being permanent. It is to be expected that a similar phase will be shown in the other genera of the family, certainly, at least, in *Dacrydium, Phyllocladus,* and *Saxegothaea.* Fig. 1A in Doyle and Looby's paper (1939) of the proembryo in the latter genus probably shows this phase, but the genus needs reinvestigation to make the point definite. In future descriptions of podocarpean proembryos a clean distinction must be kept between *relict* nuclei proper and the nuclei of this *open-cell tier.*

3. Within the sub-genus *Stachycarpus* two variations in gametophyte and proembryo exist. *P. andinus, spicatus,* and *ferrugineus* possess two (at most

three) long, pointed archegonia and a tiered group of embryo cells, like that here described for *P. andinus,* with a definite terminal cap cell. The proembryo in *P. ferrugineus,* and possibly in *P. spicatus,* is derived from a 16-nucleate phase. On the other hand, *P. gracilior* and *usumbarensis* have four or five shorter and more oval archegonia, and have a proembryo derived probably from a 32-nucleate stage, in which about ten embryo cells are formed lying in one tier. Before the data on *P. andinus* were available Buchholz (1936) suggested that the *P. usumbarensis* condition was the basal podocarpean type, and that the long archegonia, 16-nucleate phase, and tiered proembryo with cap cell, were derivative, the latter two features being causally related to the development of the long, pointed archegonia. The demonstration of a 32-nucleate, perhaps even in some cases a 64-nucleate, phase in *P. andinus* seems to make this view a strained one. Although suggesting that the *development* in *P. spicatus* and *ferrugineus* is derived Buchholz agrees with the general view that *morphologically* the *P. andinus-spicatus* type is simpler. If, then, development in *P. andinus* be taken as primitive a clear sequence follows. It has already been emphasized (Looby and Doyle, 1939, 1944) that the gametophytes of *P. andinus* (and probably also *P. spicatus* and *ferrugineus*) are almost identical with those of *Saxegothaea,* and at least closely resemble those of *Phyllocladus* and some species of *Dacrydium.* The common occurrence in these different types of the longish, pointed archegonia suggests that it is a retention of a basal form. In all also is the tiered proembryo with cap cell, except perhaps in *Dacrydium* species, which show certain signs of advance. The more direct view then would start with *P. andinus.* The primitive narrow pointed tip of the archegonia, with 32 or even 64 free nuclei, necessitated a rather coarse tiered proembryo, a condition continued in *Saxegothaea, Phyllocladus,* and some species of *Stachycarpus,* although in these the number of free nuclei may be reduced to 16. Within *Stachycarpus,* while retaining the 32-nucleate stage, a shortening of the archegonia and a widening of their base permitted the organization of the embryo cells into a single tier, a change continued directly into the Nageia section (see above). In the more advanced species of *Podocarpus* a greater precision was developed, the free nuclear number being kept at sixteen, the embryo group becoming more exact and specialized till ultimately reduced to a single cell, and the archegonia at the same time becoming more numerous and much shorter. A similar change though not quite so precise and specialized seems to characterize the genus *Dacrydium.*

4. Buchholz (1941), in his comments on the paper by Tahara (1941) already referred to, further suggests that Tahara has shown in *P. nagi,* for the first time in any podocarp, that the typical binucleate condition in the proembryo is derived from a division in each of the original cells, and not by an inclusion in pairs of the original nuclei. This, of course, must occur in *P. nagi* as in all other podocarps and could probably be seen to be fully illustrated were the paper available, but it seems necessary to point out that, two years earlier, Doyle and Looby (1939) had drawn a figure (their Fig. 1A) of *Saxegothaea* showing simultaneous divisions in what could only have been

uninucleate cells of the young proembryo. The caption under the figure ran "primary embryonic cells becoming binucleate" and it is quite clear that these authors were satisfied concerning the origin of the binucleate stage.

5. The lack of any cleavage polyembryony in the primitive embryo of *P. andinus* is further evidence in favour of the view that polyembryony is a derived condition. The form of the embryo at the tetrad stage is such, however, as to point directly to such advances as the lobing in *Saxegothaea* and the cleavage in *Dacrydium* and species of *Podocarpus*.

6. Finally it is suggested from a survey of the whole development in *P. andinus* that, in its gametophytes, proembryo, and early embryogeny, it may be taken as a prototype for the whole podocarpean complex, except for the inequality of the male cells which were probably primarily equal or subequal as they still are in the living *Saxegothaea* to-day.

SUMMARY.

A discharge of archegonial cytoplasm between the neck cells precedes fertilization. Stages in the fusion of the male and female nuclei are given. The fusion nucleus divides *in situ*, the spindle being intranuclear. The second division, also intranuclear, occurs a short way down, the four nuclei migrating to the base where successive simultaneous divisions give 32 free nuclei, of which a number up to 10, called the *relict* nuclei are not included in the subsequent embryo organization by cleavage planes. This shows an upper tier of 9–13 cells open to the archegonium and a coarsely tiered group of 11–15 embryo cells with a large terminal cell. The upper tier divides to give a prosuspensor tier and a non-permanent *open-cell* tier. This tier is probably a fundamental feature of podocarpean embryogeny. The single nuclei of the other cells each divide to give the typical *binucleate* stage, which enters into a peculiar *resting* phase during prosuspensor elongation (6 mm.). Later, double divisions in each cell followed by membrane formation give rise to 4-celled groups throughout the embryo, the *tetrad* stage. With subsequent divisions the embryo becomes homogeneous and proceeds to further development without any cleavage polyembryony.

It is suggested from a survey of the whole life-history of *P. andinus* that, in its gametophytes, proembryo, and early embryogeny, it may be taken as a prototype for the whole podocarpean complex except for the inequality of the male cells which were probably primarily equal or subequal as in *Saxegothaea*.

EXPLANATION OF PLATES.

PLATE 11.

1, 2. Cytoplasmic extrusion from archegonium before fertilization. 1 × 140, 2 × 300; June 13.
3. Male cell enters between necks. × 140; June 15.
4–7. Stages in fusion of male and female nuclei. 4 × 300; June 16. 5 × 300; June 19. 6 × 140; June 16. 7 × 140; June 15.

8. Sterile nuclei from pollen tube above fusion nucleus. × 300; June 19.
9–10. First sporophytic division. 9 × 140, 10 × 300; June 15.
11. The two nuclei passing down; remains of fusion nucleus above. × 140; June 16.
12–13. Second division. 12 × 300; June 16. 13 × 300; June 19.
14. Three of the nuclei just after telophase; location of fusion nucleus still visible above. × 140; June 19.
15. Three of the four nuclei passing to base of archegonium. × 140; June 22.
16–18. The four nuclei in the dense basal zone. × 140; June 22.

Plate 12.

19, 20. Third division. × 140; June 22.
21–24. Sections from one proembryo to show the 8 nuclei. 21–23 × 300, 24 × 140; June 22.
25, 26. Fourth division. × 140; June 22.
27–29. Stages in 16-nucleate proembryo; 27, 28 from one embryo showing 9 of the nuclei in late telophase. × 300; June 22.
30. Stage in 32-nucleate proembryo; two *relict* nuclei above. × 300; June 22.
31. Proembryo cut tangentially to show four *relict* nuclei free above. × 300; June 22.
32, 33. Nuclear organization before cleavage. × 300; 32 June 19, 33 June 24.
34. Stage in formation of cleavage membranes. × 300; June 22.
35, 38. Young walled proembryo. × 300; June 22.
36. Uninucleate embryo cells. × 600. June 22.
37. Typical resting stage. × 300; July 12.
39. Formation of prosuspensors and *open-cell* tier. × 300; June 22.
40. Division in the uninucleate embryo cells giving the binucleate stage. × 600; June 22.
41. Proembryo with *open-cell* tier still intact. × 300; June 19.
42. Nuclear type before *resting* stage. × 600; June 22.

Plate 13.

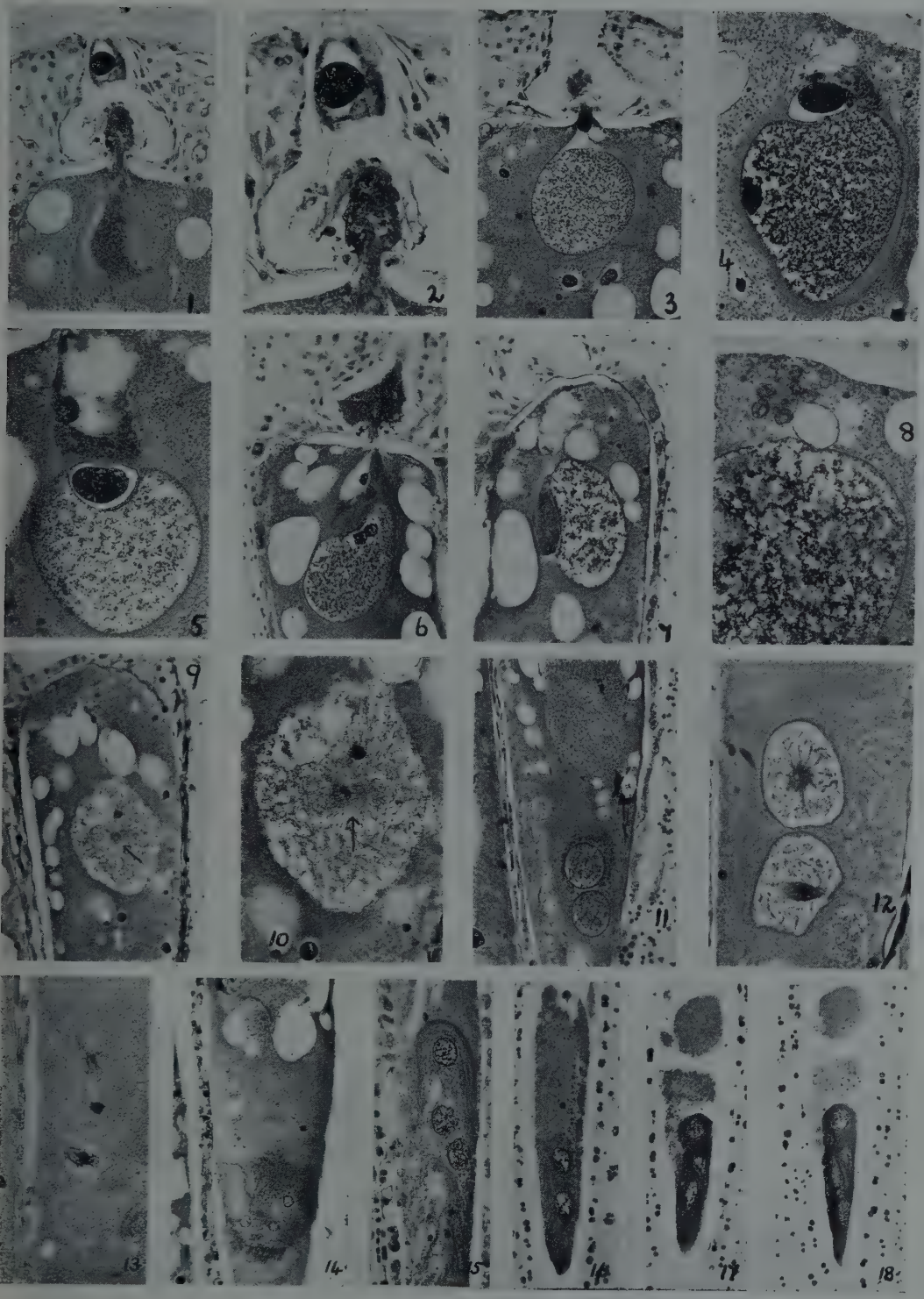
43. To show embryo nuclei after *resting* stage; degenerate cap cell below. × 600; July 12.
44. Complete dissected embryo at this stage. × 300; July 16.
45, 46. Post-tetrad divisions. × 300; 45 July 16, 46 July 20.
47–51. Serial sections of one embryo to show actual stages in paired divisions, etc., giving rise to *tetrads*. × 600; July 12.
52–54. Typical *tetrad* stage, all from one embryo. × 300; July 20.
55. To show embryo cells of 54, especially one with arrow, at higher mag. × 600; July 20.
56, 57. Dissected embryos to show tetrads. × 300; July 16.
58, 59. Intermediate stages in tetrad formation. 58 drawing × 1100 of marked cell in 52; 59 × 1250 = ditto of cell in 54 and 55, July 20.
60. Dissected embryo becoming homogeneous after tetrads, before embryonal tube formation. × 140; July 16.

PLATE 14.

61. Male and female nuclei in contact at fertilization. × 45; June 16.
62. Position of second sporophytic division. × 45; June 16.
63. Four embryonic nuclei at top of basal zone. × 140; June 22.
64. To show relative size of embryonic area to whole archegonium. × 45; June 22.
65. Irregular walled embryo. × 300; June 28.
66. Proembryo with two 'rosette' cells. × 300; June 22.
67. Proembryo probably from a 64-nucleate stage. × 300; June 22.
68. To show developing endosperm round young embryo with elongated prosuspensors. × 20; July 16.
69. Maturing embryo with embryonal tubes. × 300; July 20.
70. Embryo from 68, embryonal tubes forming and pushing up prosuspensors. × 300; July 16.

REFERENCES.

BUCHHOLZ, J. T. (1933).—Bot. Gaz., **94** (3); 579–588.
——————— (1936).—Bot. Gaz., **98** (1); 135–146.
——————— (1941).—Bot. Gaz., **103** (1); 1–37.
COKER, W. C. (1902).—Bot. Gaz., 33 (2); 89–107.
DOYLE, J., and W. J. LOOBY (1939).—Sci. Proc. Roy. Dub. Soc., **22** (11); 127–147.
LOOBY, W. J., and J. DOYLE (1939).—Sci. Proc. Roy. Dub. Soc., **22** (9); 95–117.
——————— (1944).—Sci. Proc. Roy. Dub. Soc., **23** (22) 222–237.
SINNOTT, E. W. (1913).—Ann. Bot., **27** (1); 39–82.
TAHARA, M. (1941).—Sci. Rpts. Tohoku Imp. Univ., Ser. IV, **14**; 91–98.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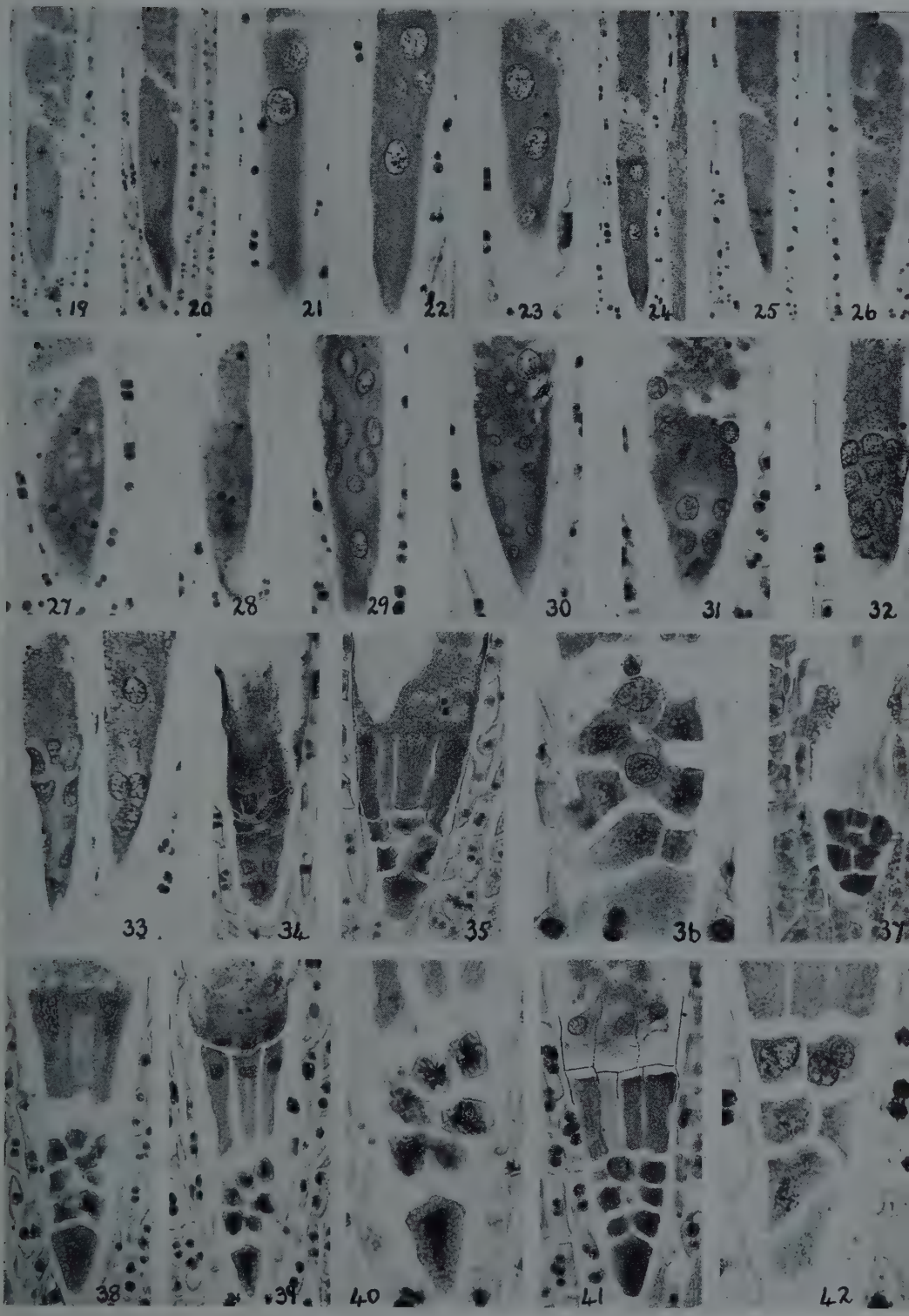
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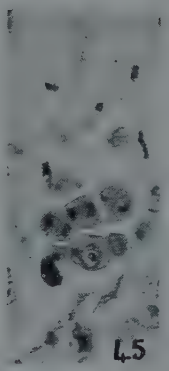
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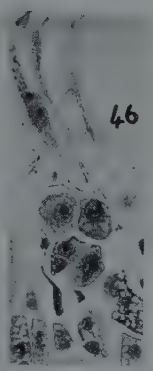
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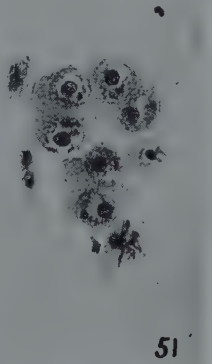
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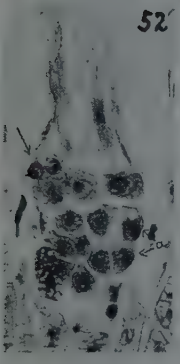
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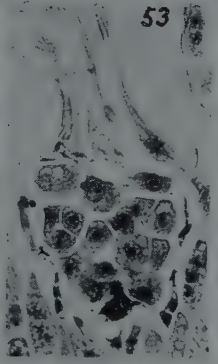
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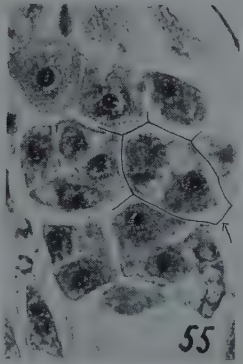
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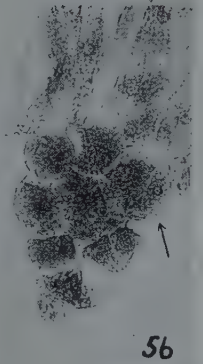
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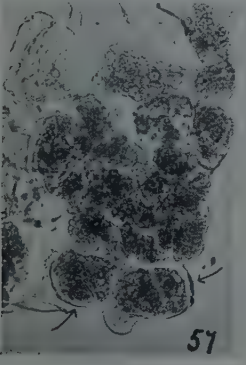
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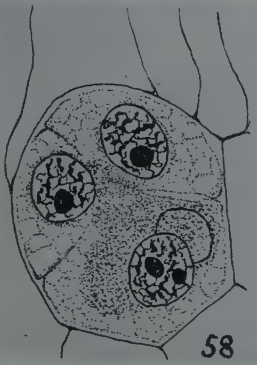
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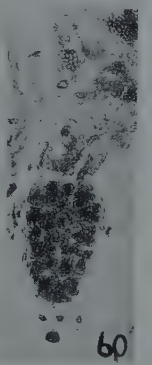
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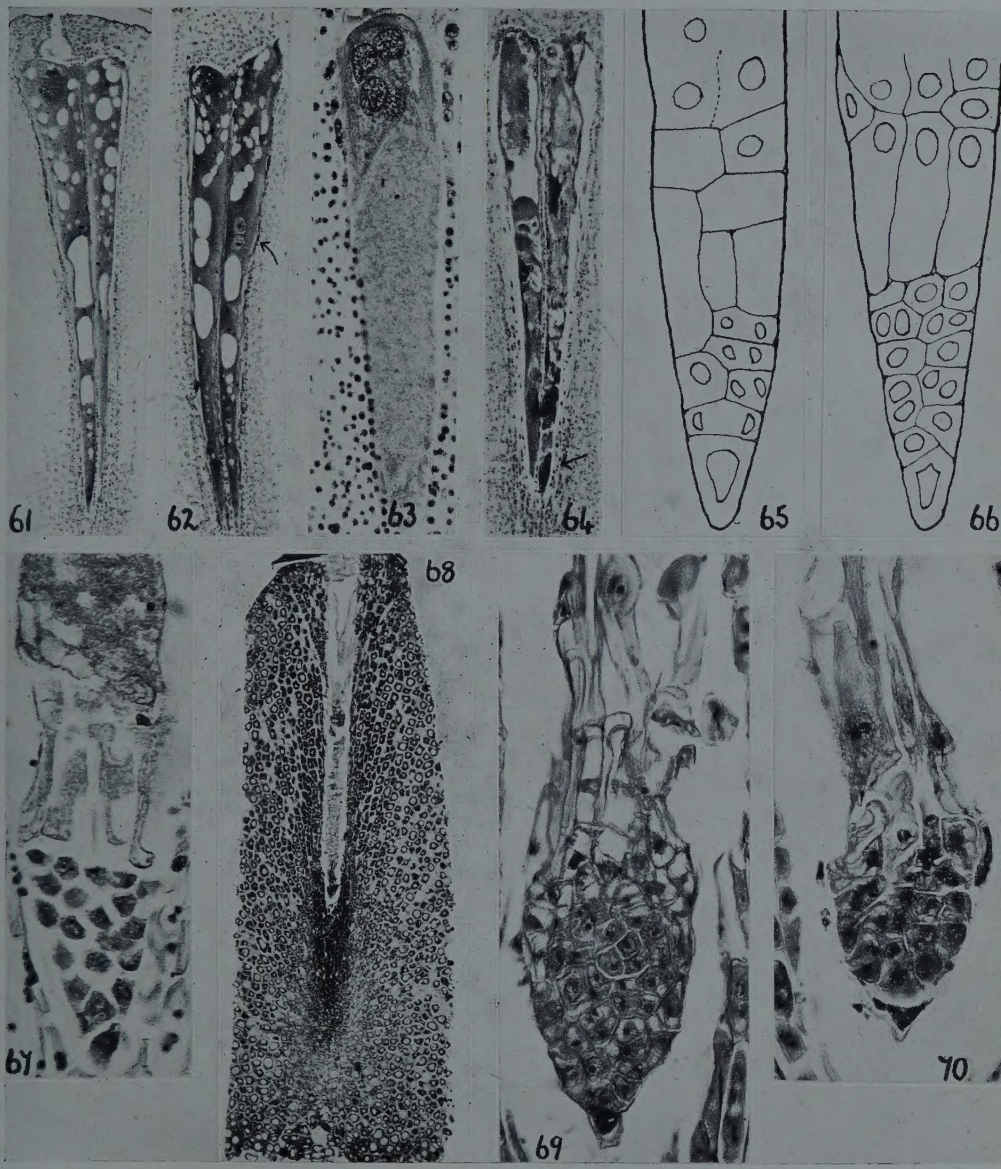
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